BACTERIAL SPOILAGE
OF
FORTIFIED WINES

J. C. M. FORNACHON

BACTERIAL SPOILAGE *of* FORTIFIED WINES

BY

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FOREWORD

In June, 1934, the Australian Wine Board resolved to ask the assistance of the Adelaide University in a careful investigation into the whole question of various diseases of wines, with the view of their prevention and cure. The Adelaide University authorities came to the aid of the Board with the utmost enthusiasm, and after several meetings of the Joint Committee a recommendation was made to the Board, that Mr. J. C. M. Fornachon, a highly qualified bacteriologist, should be appointed investigating officer, to devote his whole knowledge and energy to the work. This course was followed, and after preliminary investigations Mr. Fornachon began his work in earnest on 19th November, 1934, at the University, but it was later decided to remove the centre of activity to the Waite Agricultural Research Institute at Urrbrae, and the control was handed to the Council for Scientific and Industrial Research.

The Australian Wine Board appointed two winemakers with the necessary practical and scientific knowledge to act with Professor J. A. Prescott, Principal of the Waite Institute, and Dr. A. E. Platt, of the University of Adelaide, as a consultatory Committee with Mr. Fornachon, in the general plan of all investigations.

It was decided that the most important matter to be investigated was the occurrence of the disease known as "Tourne" among Australian winemakers, and it was to that particular disease, and later to the broader question of spoliation of wines, that Mr. Fornachon devoted his time and attention. Happily, one of his first difficulties was to find wines definitely affected with disease, and as will be found in the pages of this book, he found great difficulty in infecting sound wines. This was a well known fact with which practical winemakers were familiar, but a scientific investigator cannot accept any position on hearsay or even long experience, but must prove each point definitely as he meets it. The whole of the investigation occupied Mr. Fornachon for several years.

In 1938 it was decided that Mr. Fornachon should visit America and Europe, and return by way of South Africa; this was carried out, and Mr. Fornachon not only gained valuable experience at the University of California, but did good work during his visit there, as Professor W. V. Cruess amply acknowledged in a very appreciative letter which he sent to the Board.

After spending eleven months in California, Mr. Fornachon went on to Europe, but unfortunately the outbreak of War prevented him from completing his tour of the European vine countries, and he was hurriedly recalled to Australia; the most regrettable circumstance about this was that he could not visit the sherry making districts of Spain, where the Wine Board especially wished him to go, as the next part of his investigation was planned to be a complete enquiry into Sherry making, with special attention being given to the use of "flor" and its occurrence and effect on the Wine.

When the "spoliation of wine" investigation was completed Mr. Fornachon began on Sherry questions, and this line of research will probably occupy him for many years.

Regarding the work carried out by Mr. Fornachon, referred to in this book, one of the best and most careful and scientific of all our Australian winemakers said publicly, "If the Wine Board had done nothing but the investigation into the spoliation of wines, it would have more than justified its existence".

The credit of this great result is due to Mr. Fornachon, and he must be well satisfied that his long and patient work is crowned by such a complete and notable success.

On behalf of the Board may I take this opportunity of thanking the many members of the Scientific Staff of the Adelaide University, the President and Members of the Council of Scientific and Industrial Research, Professor Prescott and the staff of the Waite Research Institute, and many Winemakers of Australia who have helped unstintedly in the carrying out of an investigation that should be of world-wide value.

HERBERT KAY,
Chairman, Australian Wine Board.

6th August, 1943.

FOREWORD TO THE SECOND EDITION

Since the publication in 1943 of "Bacterial Spoilage of Fortified Wines", this book has become a classic in oenological research, and has been of inestimable value to the Australian wine industry. As the book is now out of print, the Australian Wine Board has undertaken to have the book reprinted for the benefit of the industry, and particularly for students of oenology and the younger technical members of the Australian wine industry.

It is characteristic of scientific research that concepts and ideas change or are modified in the light of new discoveries, and it is a tribute to J. C. M. Fornachon that his findings and their interpretation in 1943 are still very much applicable in 1969.

However, in order to bring certain statements and recommendations up to date, the opportunity has been taken in this foreword to comment briefly on certain aspects of the book, on which more recent pertinent information is available.

Sulphur dioxide. Fornachon worked mainly with total SO_2, but accurately predicted the importance of free SO_2. He and his co-workers subsequently showed that free SO_2 is considerably more important than total SO_2, and winemakers are now recommended to use free SO_2 measurements as widely as possible for both white dessert wines and white table wines. Unfortunately no reliable method is yet available for measuring free SO_2 in red wines. If dessert wines contain between 10 and 20 p.p.m. of free SO_2, depending on their pH (more SO_2 is needed at higher pH values), they are adequately protected against bacterial spoilage. The free SO_2 required in white table wines is approximately double this amount, because of the lower alcohol content of these wines.

Bacterial growth in wines. On pages 1 and 31 it is stated that growth of bacteria in Australian wines must always be regarded as dangerous. This is still current as far as fortified wines are concerned, but bacterial growth in dry red table wines has subsequently been shown to be a normal occurrence. These bacteria are responsible for the malo-lactic fermentation of red table

wines (p. 13), which was later shown by Fornachon to be widespread in Australia.

Systematic relationships of the bacteria (pp. 60-63). In collaboration with workers at the University of California, Fornachon subsequently identified *Lactobacillus* Type I as a new species which he named *Lactobacillus trichodes*. Type II was identified as *Lactobacillus hilgardii*.

Tartrate decomposing bacteria (p. 13). These were found in Australian wine for the first time in 1968. The occurrence is rare, however, and this kind of spoilage appears to be unimportant.

* * *

Following the publication of "Bacterial Spoilage of Fortified Wines" Fornachon became Officer-in-Charge of the C.S.I.R. (later C.S.I.R.O.) Section of Oenological Investigations, which arose from the research unit originally formed by the Australian Wine Board in 1934. In 1953 his second book "Studies on the Sherry Flor" was published by the Australian Wine Board. In 1955 The Australian Wine Research Institute was formed, which assumed responsibility for oenological research, and Fornachon was appointed foundation Director of Research. He filled this position with distinction until his untimely death in office on August 25, 1968, at the age of 63 years. He continued active research up to the time of his death, and published a series of important scientific papers on the microbiology of wines, a field in which he had achieved an international reputation.

B. C. Rankine,
The Australian Wine Research Institute,
1st October, 1969.

PREFACE

THE nature, cause, and control of bacterial spoilage of fortified wines have been investigated on behalf of the Australian Wine Board, and the results of the investigations are presented in this publication.

For the convenience of winemakers, the publication is divided into two parts. Part I is intended primarily for the winemakers, and comprises a general discussion of the spoilage of wine by way of introduction, followed by a summary of the results of the investigations and a discussion of the principal methods of controlling bacterial spoilage of fortified wines. Part II comprises a detailed account of the investigations which led to the conclusions presented in Part I.

In carrying out the experiments, temperatures were measured in degrees centigrade, and alcoholic strengths in per cent. of alcohol by volume, and consequently they have been expressed in these terms in Part II, but they have been converted to degrees Fahrenheit and per cent. of British proof spirit respectively in Part I since these scales are more familiar to most winemakers. The factors used for conversion are:

(a) (degrees centigrade $\times$ 9/5) + 32 = degrees Fahrenheit.

(b) per cent. of alcohol by volume $\times$ 7/4 = per cent. of proof spirit (approximately).

The writer wishes to acknowledge his indebtedness to Professor J. A. Prescott for criticism and advice during the investigations, to Mr. J. L. Williams for the benefit of his practical experience with several disinfectants in the Roseworthy Agricultural College Winery, and to Mr. A. D. Cocks for taking the photomicrographs. Acknowledgment is also made to Messrs. L. N. Salter and C. P. Haselgrove for helpful suggestions in the preparation of the manuscript, and to the many winemakers who have furnished information and samples of wines and lees and other materials.

J. C. M. FORNACHON.

Waite Agricultural Research Institute, Adelaide.

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PART I

CHAPTER I

INTRODUCTION

In contrast to many other articles of human diet, wine is not a suitable medium for the growth of a wide range of micro-organisms. Many different kinds of bacteria as well as yeasts and moulds may find their way into wine, but, fortunately for the winemaker, the majority of these organisms are unable to multiply because of the acidity and the alcohol content of the wine. A few types of bacteria are able to flourish in wine, however, and these are of great importance to the winemaker since they bring about changes in the composition of the wine which usually lower its quality and often render it undrinkable. Some bacteria may, under certain conditions, bring about an improvement in flavour by decomposing malic acid and so lowering the acidity of wine; in fact these malo-lactic bacteria, as they are called, are actually encouraged to grow in certain light dry European wines which would otherwise be excessively sour owing to the presence of large amounts of malic acid. More usually, however, the growth of bacteria in wine is accompanied by spoilage and, under Australian conditions, must always be regarded as dangerous.

Bacterial spoilage of wine is by no means a new problem, but has been the subject of a considerable amount of research in Europe during the last eighty years. Until recently, however, practically all of this research was concerned with unfortified wines, for these were the only wines in which evidence of bacterial growth was common. It was generally assumed in most winemaking countries that wines containing more than 30 per cent. of proof spirit were immune from bacterial attack, but during the last twenty years bacterial spoilage of fortified wines containing 35 to 37 per cent. of proof spirit has been reported in Southern Europe, South Africa, California, and Australia.

It is natural that the winemaker should turn to the standard oenological text books in search of information on spoilage problems. As the discussions of bacterial spoilage contained in such text books are concerned almost entirely with unfortified wines under European conditions, however, the winemaker encounters difficulties concerning the relationship between the spoilage of fortified wines and the various types of spoilage or diseases of wine described by European writers. A considerable amount of confusion has arisen concerning the relationships between different diseases of wine, and the name 'tourne' is often applied to the spoilage of fortified wines because of the similarity between the microscopic appearance of the lees from infected fortified wines and the diagrams and photographs in European text books.

Relationships Between Different Types of Spoilage. In order to understand the relationships between different types of spoilage it is necessary to distinguish between the results and the causative organism. It should be clearly understood that the names 'tourne,' 'pousse,' and 'amertume' used in oenological text books are *not names of bacteria,* but are names given to certain changes which occur in wines as a result of bacterial activity. Such names refer to the effects of the bacteria and not to the bacteria themselves. Thus, questions concerning the similarity between the spoilage of fortified wines and 'tourne' can be answered only by consideration of the changes occurring in the infected wines.

The meanings of some of the names used to describe different diseases of wine in Europe have changed somewhat during the last eighty years. Such types of spoilage as 'tourne,' 'pousse,' and 'amertume' were referred to by these names in France long before anything was known about the chemical changes occurring in the wines or the cause of the spoilage. Originally these names denoted only the more obvious changes in the appearance, flavour, and aroma of the wines and were used in much the same way as we use the terms sourness, gasiness, and bitterness to indicate different types of spoilage occurring in milk. During the latter half of the 19th century the spoilage of wine was investigated by a number of workers in France and other countries and the characteristics

of the different types of spoilage were gradually determined and described. As might be expected, however, in view of the complexity of the problem, there is often some lack of agreement between the descriptions given by different investigators.

Since the name 'tourne' is commonly applied in Australia to the spoilage of fortified wines it might be of interest to consider briefly some of the descriptions of 'tourne' published by European writers. Béchamp in 1862 referred to the disappearance of glycerine from 'vins tournes,' while Nickles, in the same year, claimed that 'tourne' was characterized by the formation of propionic acid by decomposition of tartrates in the wine. This latter view was subsequently supported by the eminent French scientist Duclaux and has since been widely accepted. Gautier on the other hand, in 1878, described a type of spoilage commonly known as 'tourne' in the south of France, and which he claimed was characterized by the decomposition of tartrates and the formation of acetic, tartronic, and lactic acids. Several other descriptions of 'tourne' have also been published, but it should be understood that all of the early descriptions were based on the results of analyses carried out on wines which had become unsound under industrial conditions, for at that time no satisfactory method of inducing spoilage under controlled conditions had been developed. Thus although differences were noticed between the compositions of sound and unsound wines, it was not known whether these differences were entirely due to the spoilage or whether they represented differences in the original wines. Much of the confusion concerning 'tourne' was eventually dispelled by the researches of Müller-Thurgau and Osterwalder which were published in 1919. By means of carefully controlled experiments and using pure cultures of bacteria, these workers showed that a rod-shaped bacterium which they called *Bacterium tartarophthorum,* was able to cause 'tourne' when growing in a dry wine. In such a wine the bacterium attacked the tartrates and often the glycerine as well. The tartrates were decomposed to acetic acid and carbon dioxide while the glycerine was decomposed to lactic, acetic, and propionic acids and carbon dioxide. This description of

'tourne' is now accepted by most leading authorities, but unfortunately some of the early descriptions are still quoted in oenological text books. The custom of quoting the results obtained by early investigators, without taking into account the conditions under which the results were obtained, or the status of oenological research at the time that the investigations were carried out, is largely responsible for the lack of agreement between the accounts of certain diseases of wine included in different text books.

It does not seem desirable to include here a detailed description of each of the types of bacterial spoilage known in Europe, but the foregoing remarks should be sufficient to indicate how terms such as 'tourne' which were originally used in a vague descriptive sense, have gradually been defined chemically and have acquired a definite meaning. The changes which characterize the bacterial spoilage of Australian fortified wines are very different from those mentioned in the preceding paragraph and consequently this type of spoilage is not the same as that known as 'tourne' in Europe. The spoilage of fortified wines will be discussed more fully, however, in the following chapter.

The Types of Bacteria which Cause Spoilage of Wines. The relationships between the causative bacteria appear to be even less clearly understood than the characteristics of the different types of spoilage. Before discussing the relationship between the bacteria causing spoilage in fortified wines and those described by other workers, however, we will consider very briefly the means by which bacteria are identified and compared and the types of bacteria which are able to cause deterioration in wines. In the first place it must be realized that the microscope has its limitations as a means of identifying bacteria. Although this instrument is of immense value to the wine-maker in enabling him to determine whether bacteria are present in his wine and to distinguish one broad group from another, the organisms cannot be identified with any degree of certainty simply by a microscopical examination. Bacteria which have very different characteristics may appear to be identical when examined under the microscope and, on the other hand, a single strain of a bacterium may exhibit quite

different appearances when growing under different conditions. The identification sometimes requires weeks or even months of careful work, for it is necessary to isolate the organism in pure culture and to determine not only its size and shape, but also the way it stains with different dyes, the way in which it behaves under different experimental conditions, the food substances it is able to use, and the waste products which it forms.

The classical researches of Pasteur established the fact that bacteria were present in many unsound wines and also furnished descriptions of their microscopic appearance, but it must be remembered that the methods which are now used for isolating and identifying micro-organisms had not been developed at that time. Consequently we find in the early literature many descriptions of the appearance of bacteria found in wines but no detailed accounts of their other characteristics. Such descriptions are of very little value as a means of identifying the bacteria or comparing those described by different workers. It was not until the close of the 19th century that Gayon and Dubourg and also Laborde actually isolated bacteria causing diseases in wine not left exposed to the air, and described them in sufficient detail to enable us to recognize them. It was chiefly the work of Müller-Thurgau and Osterwalder, however, which established wine bacteriology on a sound scientific basis, and our present knowledge of the characteristics of the different spoilage bacteria is largely due to the careful and thorough researches of these investigators. The early literature contains many references to bacteria which decompose tartaric acid in wine, but the first recognizable description of such an organism appears to be that of *Bacterium tartarophthorum*, published by Müller-Thurgau and Osterwalder in 1919.

Here it may be pointed out that the fact that a bacterium is isolated from a spoiled wine does not necessarily indicate that it is the cause of the spoilage. In order to prove such a relationship it must be shown that the bacterium, after being isolated and sown in a sterile sound wine, brings about the same type of spoilage as occurred in the original wine. Some confusion has been caused by failure to observe this important requirement, and it seems highly unlikely that some of the bac-

teria which were isolated from unsound wines by early workers were really the cause of the trouble.

The bacteria which have so far been proved responsible for causing spoilage in wine and which have been described in sufficient detail to allow them to be recognized, belong to the following genera: *Acetobacter*, *Micrococcus*, *Leuconostoc*, and *Lactobacillus*.

The *Acetobacter* are the acetic acid or vinegar bacteria which are used in making vinegar and which cause souring of light wines left exposed to the air.

The genera *Micrococcus* and *Leuconostoc* include a number of species of bacteria which are spherical in shape. It appears that bacteria belonging to these groups sometimes cause deterioration of wines in Europe. Occasionally they have been isolated from Australian wines, but under our conditions they have not been shown to be of sufficient importance to warrant special investigation.

The genus *Lactobacillus* comprises the rod-shaped lactic acid bacteria, some of which are of great importance to the winemaker. Bacteria belonging to this genus are widely distributed in nature and some of them play important parts in such diverse processes as the maturing of cheese, the making of silage, the souring of distillery mash, and the spoilage of wine and beer. Some species of *Lactobacillus* form only lactic acid from sugars, whereas other species form appreciable amounts of other products as well as lactic acid. Species belonging to this second group form lactic acid, acetic acid, alcohol, and carbon dioxide from dextrose (glucose), and from levulose (fructose) they form lactic acid, acetic acid, carbon dioxide, and mannitol (mannite). Some of the species belonging to this second group are the most important bacteria causing spoilage of wines.

The lactobacilli attack other sugars besides dextrose and levulose, and since species differ in their ability to attack different sugars, some sugars are useful as a means of identifying the bacteria. Some species are also able to attack certain organic acids when fermentable sugars are not available. The so-called malo-lactic fermentation which occurs in certain European wines consists of bacterial decomposition of malic

acid to lactic acid and carbon dioxide and may be caused by several different species of *Lactobacillus*. The tartrate decomposing organism described by Müller-Thurgau and Osterwalder under the name of *Bacterium tartarophthorum* is also a species of *Lactobacillus* according to the description which these workers published in 1919. It should be noted, however, that the bacteria which decompose these organic acids in wine do so only when there is little or no fermentable sugar present. In a sweet wine they attack the sugars dextrose and levulose and form lactic acid, acetic acid, carbon dioxide, and mannitol. Thus the term 'mannitic bacteria' which frequently occurs in oenological text books, is not confined to any one species but includes all of the gas-producing species of *Lactobacillus* since they all form mannitol from levulose.

It is evident that the type of change which occurs in a wine is not entirely dependent on the species of bacterium present, but that it depends also on the composition of the wine. Only some species are able to attack tartaric acid and malic acid and so cause 'tourne' and malo-lactic fermentation. If the wine contains an appreciable amount of fermentable sugar, however, then the bacteria attack this in preference to the organic acids and so behave like other species of this group and cause lactic-acid spoilage. Thus a single species of bacterium may cause different types of spoilage in different wines, while on the other hand several different species may cause the same type of spoilage if growing in similar wines.

CHAPTER II

THE SPOILAGE OF AUSTRALIAN FORTIFIED WINES AND THE BACTERIA WHICH CAUSE IT

This spoilage is not confined to any one type of wine, nor to the wines of any particular district, although it does seem to be rather more common in white than in red wines. Of the unsound wines which have been examined during these investigations, a greater number of the white wines had been made from Doradillo and Gordo Blanco than from other varieties of grapes, while a large number of the red wines had been made from the Grenache variety.

This is not necessarily because of any special property of the wines made from these varieties, however, for they represent a large proportion of the whole of the fortified wine made in Australia.

As this publication is chiefly concerned with the spoilage of fortified wines, the characteristics of this type of spoilage will be considered first and then a brief description of the bacteria responsible will be given. Later it will be pointed out that some of these bacteria also cause deterioration of dry wines and musts.

1. Spoilage of Fortified Wines

The signs of bacterial spoilage in fortified wines vary somewhat according to the composition of the wine and the development of the bacteria. Usually, but not always, the most obvious sign is the change which occurs in the appearance of the wine. Some wines remain almost clear except for a flocculent deposit, sometimes resembling a mass of wet cotton-wool, which is formed on the bottom of the container. More usually, however, the infected wine becomes dull and cloudy and when a bottle or a glass of such wine is held up to the light and shaken with a rotary motion a peculiar silky, streaming appearance is at once obvious. This appearance is what French

writers refer to as 'ondes soyeuses' when they describe wines affected by 'tourne,' and it is likely that this similarity between the appearance of infected fortified wines and that of 'vins tournes' is partly responsible for the frequent use of the term 'tourne' in connection with the spoilage of fortified wines. The appearance referred to is not confined to any one type of disease, however, nor is it characteristic of any one species of bacterium. It is a physical effect caused by the bacterial rods becoming aligned in chains, and is most apparent with bacteria which occur as long fine rods and chains. Actually a very similar silky, streaming appearance is sometimes seen in suspensions of needle-shaped crystals, but when it is noticed in wine it is usually caused by rod-shaped bacteria and should be taken as an indication of infection.

Often the appearance of the wine is greatly affected before other signs of deterioration have become very obvious. In advanced stages, however, the wines acquire objectionable acid flavours and odours which differ in different wines. In some, but not in all affected wines, a flavour and odour reminiscent of mice is noticeable. This characteristic is commonly referred to as 'mousiness' and has been noticed in different types of wine and in several different countries. Müller-Thurgau and Osterwalder thought that mousiness was characteristic of wines attacked by *Bacterium mannitopoeum*, but in 1938 Vaughn showed that mousiness is not characteristic of wines attacked by any one species, but may be caused by several different bacteria.

The most obvious change which occurs in the composition of the wine during spoilage is an increase in both volatile and fixed acidity due to bacterial decomposition of the sugars present in the wines, and the formation of acetic and lactic acids. As the fixed acidity of the wines in which the bacteria develop most readily is often very low at the time of infection, however, the unsound wines are not usually characterized by a very high fixed acidity. Carbon dioxide gas is another product formed by the bacteria, and when they develop in bottled wines these may become heavily charged with gas. Mannitol (mannite) is also formed, but is usually not present in large amounts in spoiled fortified wines.

The type of spoilage which has just been described is similar to the disease of South African fortified wines described by Niehaus and of Californian fortified wines described by Cruess, but is not the same as the spoilage known as 'tourne' in Europe. As has already been pointed out, 'tourne' is characterized by decomposition of tartrates, increased volatile acidity, and *decreased* fixed acidity, whereas the spoilage which occurs in fortified wines is characterized by decomposition of sugar and increase in *both* volatile *and* fixed acidity. Of the various diseases of wine which have been described by European writers, the lactic acid spoilage described by Müller-Thurgau and Osterwalder in 1913 and 1918 resembles the spoilage under discussion most closely, but the alcohol content of the wine mentioned by Müller-Thurgau and Osterwalder was much lower than that of Australian fortified wines.

2. The Bacteria

The organisms responsible for the deterioration of fortified wines are rod-shaped bacteria belonging to the genus *Lactobacillus*. It has been shown that these bacteria are widely distributed, and it seems likely that they are present in all Australian wineries. They have been found in practically all of a large number of gross lees samples which have been examined, and although they were often present in insufficient numbers to be detected by means of the microscope, their presence could be demonstrated in other ways. When present in such small numbers the bacteria do not cause trouble, but it is necessary that the winemaker should realize that they are usually present in sound wines and lees and that they are ready to multiply rapidly and cause serious damage as soon as conditions become favourable for their growth. The significance of this particular point in relation to the control of spoilage will be dealt with more fully in a later chapter.

In badly affected wines the bacteria are present in enormous numbers and they may be seen quite readily by examining a drop of the wine or lees under a microscope with a magnification of about 500 diameters. The appearance of the bacteria when found in different wines and lees varies considerably.

Sometimes they appear as single rods or pairs of rods joined end to end and often bent at an obtuse angle, sometimes they appear as long or short chains of rods and sometimes as long thread-like filaments which may be interwoven in a tangled mass and intermingled with particles of colouring matter, tartrate crystals, and other lees material. The long tangled filaments are particularly common in the lees of wines which have formed the flocculent deposit referred to in a previous paragraph.

The lactobacilli which cause spoilage of fortified wines have been classified into five types during these investigations and they are described in detail in Part II. Type I differs from previously described species, while Types III and IV are varieties of *Lactobacillus buchneri (Bacterium mannitopoeum*—Müller-Thurgau) and Types II and V appear to be closely related to *Lactobacillus fermentum (Bacterium intermedium*—Müller-Thurgau and Osterwalder).

The five types resemble one another in many respects, for they are all rod-shaped and may occur as single rods, pairs of rods, chains of rods, or long fine filaments. They multiply by the process known as transverse fission, which simply means that a rod divides transversely and becomes two rods. None of them form spores and none of them have any means of independent locomotion, although it is important to remember that when growing in wine they are carried from one position to another by movement of the wine, rising bubbles of gas, and by gravity. Sometimes when a drop of infected wine is examined under the microscope the bacteria do appear to be moving, but this is due to movement of the liquid and other factors acting on the bacteria and not to any independent action on the part of the bacteria.

By means of special laboratory tests, the different types of bacteria can be identified, but they cannot be distinguished from one another with certainty by a microscopical examination alone. The various rods, chains of rods, and filaments which may be seen in different wines and lees do not necessarily mean that different types of bacteria are present, for the appearance of the bacteria varies according to the conditions under which they are growing. This point is well illustrated

by the photomicrographs. Figs. 1, 2, and 3 show the different appearances which a single strain of a bacterium may present when grown under different conditions, while Figs. 4, 5, 6, and 7 show the similarity in the appearance of four different types of bacteria grown under similar conditions.

The five types of bacteria all cause the same type of spoilage when growing in a sweet wine, for they all attack the sugars dextrose and levulose and form acetic acid, lactic acid, mannitol, and carbon dioxide. Type I is more tolerant towards alcohol than the other types, and for this reason is the most important organism causing deterioration in wines fortified to strengths of 35 per cent. proof spirit or over. In less highly fortified wines, however, some of the other types are even more destructive than Type I.

3. Spoilage of Dry Wines

The term 'dry' as applied to wine is rather inexact, but broadly speaking, a dry wine may be defined as one in which the presence of sugar is not obvious to the palate. Nearly all wines contain some sugar, and the amount which may be present and still not give the wine a perceptibly sweet taste varies in different wines.

Some wines which are nominally 'dry' contain appreciable amounts of residual sugar as a result of 'stuck' fermentations. Such wines are notoriously subject to spoilage for they usually have quite a low alcohol content and consequently the bacteria grow very readily in them if other conditions are favourable. The type of change which occurs in these wines is essentially similar to that which occurs in fortified sweet wines, but it frequently develops more rapidly. Many of the fortified dry white wines also contain quite appreciable amounts of sugar and undergo similar spoilage to that which has already been described.

In light dry wines containing not more than 0·1 per cent. of sugar the bacteria are able to grow sufficiently to alter the appearance of the wine considerably, but usually such wines are not appreciably altered in composition. During the growth of the bacteria the wines become dull and cloudy and may remain in this condition for weeks or months, but eventually

the bacteria settle to the bottom of the containers and the wines may ultimately show little evidence of bacterial development. In wines which contain rather larger amounts of residual sugar the evidence of spoilage is usually more apparent, and when the sugar content exceeds 0·2 to 0·3 per cent., as is the case in some of the wine used as 'flor' sherry material, bacterial growth may cause quite a pronounced 'off' flavour and odour and an appreciable increase in volatile acidity. Even when the composition of the wine is not appreciably affected, the loss of condition which accompanies bacterial growth is in itself undesirable and may be a serious matter if it occurs in bottled wines, particularly if these have already left the hands of the winemaker.

Reference has already been made to the malo-lactic fermentation which occurs in certain European wines. As far as the writer is aware, malo-lactic fermentation has not been observed in Australian commercial wines. This may be due to the low malic acid content of the majority of our wines, but it is certainly not due to lack of bacteria capable of causing a malo-lactic fermentation. Several strains of lactobacilli isolated from Australian wines bring about a malo-lactic fermentation when grown in dry wines to which malic acid has previously been added.

In Europe some of the most serious spoilage is caused by bacterial decomposition of tartrates and glycerine in dry wines. None of the strains of bacteria isolated from Australian wines, however, could be induced to attack tartrates or glycerine either in wines or in artificial culture media.

4. Spoilage Due to Growth of the Bacteria in Must

Although the bacterium referred to in this publication as Type I is very destructive in fortified wines, it appears to be unable to grow in grape must and so cause spoilage before the wine is made. Many strains of the other types, notably Types III, IV, and V, grow very readily in must, however, and may cause serious spoilage before or during the primary fermentation. In this way fortified wines can be ruined before they are actually fortified, and this may be brought about by bacteria which do not have a very high tolerance towards

alcohol. Growth of the spoilage bacteria in the fermentation tank usually results in a wine which has a high volatile acidity and which remains dull and cloudy. If such material is not fortified the high volatile acidity often interferes with the activity of the yeasts and the must does not 'ferment out dry.' Thus the resulting wine contains an appreciable amount of residual sugar, has a low alcohol content, and is therefore particularly susceptible to further spoilage in the storage vats.

CHAPTER III

FACTORS AFFECTING THE GROWTH OF THE BACTERIA IN WINES

Most winemakers realize that some wines are much more liable to bacterial attack than others, and it has been found in the laboratory that many wines remain sound even after being inoculated with spoilage bacteria and incubated, whereas others deteriorate quite rapidly. Such differences in the susceptibility of wines to bacterial attack are obviously of very great importance in relation to methods of preventing spoilage, and for this reason a large part of these investigations has been concerned with the question of susceptibility of wines. By studying the effects of various factors on the growth of the bacteria, it has been shown how differences in the composition of wines are related to differences in susceptibility and how these differences may be controlled and thus utilized in the prevention of spoilage. The methods advocated for preventing bacterial attack will be discussed in the next chapter, but in order that the winemaker may apply these methods intelligently it is desirable that he should know something of the factors which favour or inhibit the growth of bacteria in his wines.

It has been found that the spoilage bacteria are very exacting in their growth requirements, that is to say, they flourish only when conditions are just right. In many respects the growth requirements of the five types of bacteria are similar, but some differences have been noticed. Type I can grow at higher alcoholic strengths than the other types, but in some respects is more fastidious than the others, and does not grow in must whereas some of the others do. Other differences in the effects of some factors on the growth of the different bacteria will be mentioned as each of the factors is discussed.

1. Acidity of the Wines

Probably most winemakers are aware that wines with a high acidity are less liable to bacterial attack than wines with a low acidity, but it is less generally realized that the effect of acidity on bacterial growth depends on the pH value rather than the titratable acidity of the wine.

Since many winemakers may be unfamiliar with the term 'pH' and are accustomed to determining only the titratable acidity or the total amount of acid present, the following simple explanation is offered in the hope that it will assist them to gain a better understanding of this very important subject.

Acidity and pH.

When any acid goes into solution, some of it splits up or dissociates, and liberates electrically charged particles of hydrogen, or hydrogen ions as they are called. These hydrogen ions give to the substance its acid properties and the acid activity depends on the concentration of hydrogen ions in the solution.

The pH scale provides a measure of the amount of dissociated acid in the solution or of the concentration of hydrogen ions. Titratable acidity, on the other hand, provides a measure of the whole of the acid present, i.e., both dissociated and undissociated.

The proportion of the acid which splits up or dissociates depends on the kind of acid, the amounts and kinds of other substances present, and other factors. Thus two solutions may have the same titratable acidity (or contain the same total amounts of acid) and yet contain very different amounts of hydrogen ions and so have very different acid activities or pH values.

As an illustration let us consider a solution of hydrochloric acid of common laboratory strength (one-tenth normal) and a solution of acetic acid of the same strength. If similar quantities of these two solutions are titrated in the ordinary way with a standard alkali solution (e.g., caustic soda), they will require the same amounts of the alkali to neutralize them. Thus the two solutions have the same titratable acidity. However, almost all of the hydrochloric acid is dissociated whereas

only a very small portion of the acetic acid is dissociated. Thus the concentration of hydrogen ions in the hydrochloric acid is nearly a hundred times as great as in the acetic acid solution and consequently the pH values of the two solutions are very different.

The accompanying diagram shows the pH scale and the pH values of some common substances. As can be seen, the scale is graduated from zero to 14 and is so arranged that a low pH value signifies a high degree of acidity and a high pH value signifies a low degree of acidity. pH 7 is the neutral point and pH values above 7 signify alkalinity.

Another important feature of the pH scale is that each unit on the scale represents a tenfold increase or decrease, e.g., pH 1 is ten times as acid as pH 2, pH 2 is ten times as acid as pH 3, pH 3 is ten times as acid as pH 4, and so on.

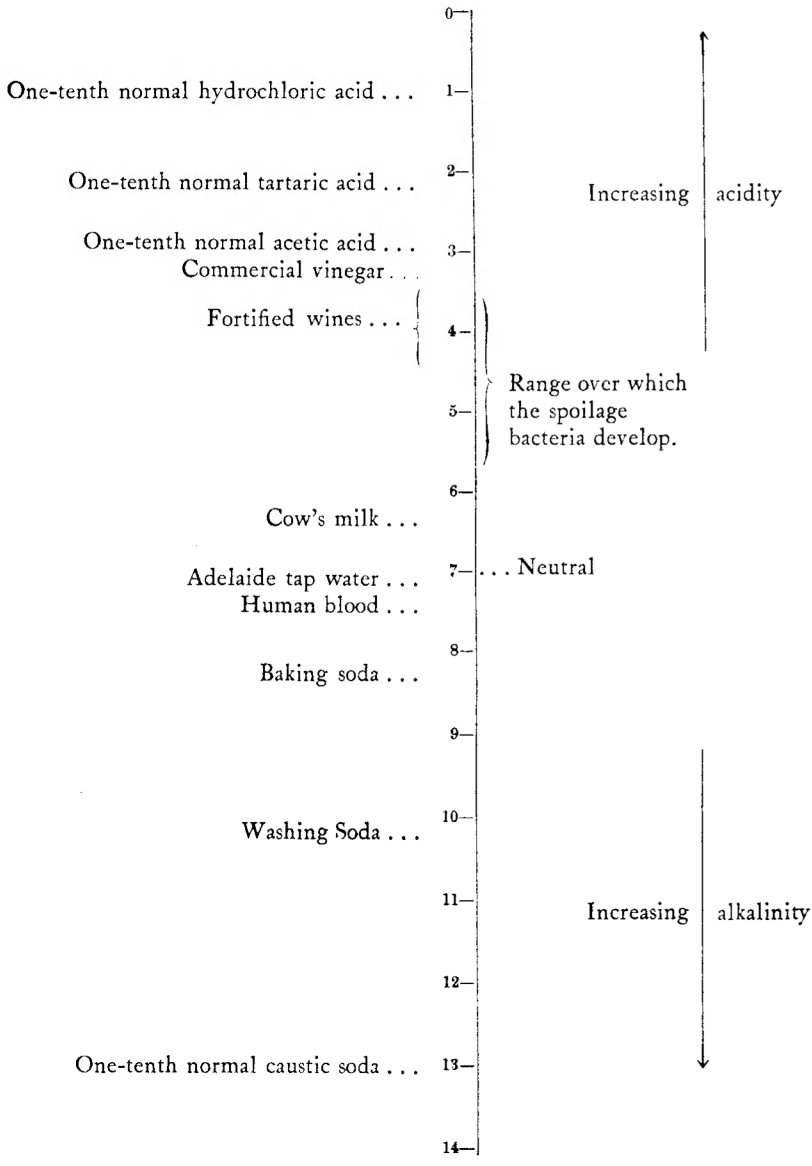
One is frequently asked such questions as 'What is the pH value of a wine which contains 5·5 grms. of tartaric acid per litre?' or 'How much acid must be added to a wine to lower the pH to 3·6 if the titratable acidity is 4 grms. per litre?' Unfortunately such questions cannot be answered unless the necessary pH determinations are carried out on the particular wine in question. It has already been pointed out that the pH value of a wine or any other solution depends on other factors besides the amount of acid present. When these other factors are not fully known, as in wine, it is not possible to prepare a simple table by means of which pH values may be translated into titratable acidity.

Several methods are available for determining the pH of solutions, but bearing in mind the cost of the necessary apparatus and the degree of accuracy required, the quinhydrone electrode method is probably the most suitable for determining the pH values of wine. Suitable pH outfits which render the determination of pH values comparatively simple can be purchased from any of the leading dealers in chemical equipment.

The pH values of Australian fortified wines vary from about 3·3 to 4·3, but the majority of them are between 3·5 and 4·0. Other things being equal, the lower the pH value (higher the degree of acidity) the less liable the wine will be to bacterial

THE pH SCALE

Showing the pH Values of Some Common Substances.



attack. The lowest pH value (highest degree of acidity) at which the bacteria are able to grow in wines containing 35 per cent. of proof spirit appears to be very close to pH 3·6. In less highly fortified wines, however, some strains of the bacteria (particularly of Types III, IV, and V) are able to tolerate a somewhat lower pH and may develop at pH values as low as 3·4.

2. Temperature

There seems to be a fairly widespread belief that the spoilage bacteria require high temperatures for their growth, but this has not been found to be so. In fortified wines the bacteria grow most rapidly at temperatures between 20° and 25° C. (68° and 77° F.) and few of them are able to grow in these wines when the temperature exceeds 28° to 29° C. (82·4° to 84·2° F.). Bacterial growth at temperatures below 20° C. (68° F.) is usually rather slow, but is quite appreciable, and wines stored at temperatures in the vicinity of 15° C. (59 F.) may undergo considerable deterioration over a long period. In wines containing less than about 26 per cent. of proof spirit and in musts, some strains of the bacteria (particularly strains of Types III, IV, and V) grow very rapidly at temperatures which are higher than those most favourable for the growth in fortified wines. In these wines of low alcohol content and in musts, bacterial growth is often most rapid when the temperature is between 30° and 35° C. (86° and 95° F.), but here also spoilage may be very considerable at much lower temperatures. High temperatures have an important effect on spoilage, however, quite apart from their direct effect on the growth of the bacteria, for high temperatures during the primary fermentation and during storage up till the time of the first racking render the wine more liable to bacterial attack subsequently (see next paragraph).

3. Accessory Growth Substances

Probably most people are now familiar with the importance of vitamins in human nutrition. Although we require only very small amounts of the various vitamins, their presence in

our diet is essential if we are to remain in normal health. It is an interesting fact that many bacteria, too, require small amounts of certain vitamin-like substances for normal growth. These vitamin-like substances required by bacteria are called accessory growth substances, for although their presence is essential for normal growth they are accessory or additional to the ordinary nutrients required by the bacteria.

The spoilage bacteria, like other lactobacilli, require several such substances, some of which are present in normal grape juice and some of which are not. An interesting feature of the requirements of bacteria for accessory growth substances is that quite closely related species differ in their requirements, and it is probably for this reason that we find some of the spoilage bacteria able to grow quite readily in must while others fail to do so.

All of the accessory growth substances required by the spoilage bacteria are present in yeasts, and it has been found that the addition of small amounts of a specially prepared yeast extract to many wines enables bacteria to grow in them quite vigorously, when they otherwise failed to grow in these wines at all. Thus one of the reasons why some wines remain sound even when inoculated with bacteria is that they do not contain all the accessory growth substances required by the bacteria.

As has already been pointed out, yeast cells are a rich source of the accessory growth substances required by the spoilage bacteria. Now under some circumstances the yeast cells which are present in fermenting must, in young wine, and in the gross lees, undergo a special kind of decomposition which is called autolysis. As a result, the accessory growth substances are liberated in the wine and so the wine becomes more favourable for the growth of the bacteria. Autolysis of yeasts is favoured by high temperatures and high pH (low acidity). It has been found that, other things being equal, wine made from must with a high pH is more susceptible to bacterial attack than wine made from must with a low pH; wine made at a high temperature is more susceptible than wine made at a low temperature, and wine left on the gross lees for a long period is more susceptible than early-racked wine. Thus high

temperatures and high pH values have an important effect on the susceptibility of the wine quite apart from the direct effects of temperature and acidity on the growth of the bacteria.

4. Nitrogen

It is sometimes claimed that the amount of nitrogen present in wines is important in relation to bacterial spoilage. It has been found, however, that neither the total amount of nitrogen nor the amount of several different forms of nitrogen (ammonia, amino-nitrogen, protein) is a reliable indication of the susceptibility of wine to attack. Quite vigorous bacterial growth has been obtained in a number of laboratory-made wines which contained much less nitrogen than the majority of Australian fortified wines, and it is therefore unlikely that many wines contain insufficient nitrogen for bacterial growth. It is possible, however, that some wines contain insufficient of one or more particular nitrogenous compounds (particular amino acids) for bacterial growth, and since these are present in autolysed yeast, the growth-promoting effect of autolysed yeast may be due in part to the presence of particular amino-acids as well as the accessory growth substances discussed in the previous paragraph.

5. Sugar

The chief products formed by the spoilage bacteria, lactic acid, acetic acid, carbon dioxide, and mannitol, all originate from the sugars present in the wine, but variations, within normal limits, in the amount of sugar present in fortified wines have not been found to affect bacterial growth. In dry wines, however, lack of sugar is often the factor which limits the growth of the bacteria and the extent of the spoilage. In light dry wines containing not more than 0·1 per cent. of sugar, spoilage is usually confined to loss of condition. Wines containing larger amounts of residual sugar support a more vigorous growth of bacteria and show evidence of spoilage in flavour and odour, while wines containing about one per cent. or more residual sugar as a result of 'stuck' fermentations are usually very susceptible to bacterial attack and often suffer serious deterioration.

6. Alcohol

The chief reasons for fortification are to arrest the primary fermentation while the wine still contains some sugar and to protect this sugar from subsequent attack by yeasts and bacteria. Long experience has shown the makers of sweet wines that fortifying to a strength of about 35 per cent. of proof spirit protects the sugar in the wine against practically all yeasts and most bacteria, and for many years it was generally believed that wines containing 35 per cent. of proof spirit were immune from bacterial attack. We now know that such wines are not all immune from bacterial attack by any means, but, at the same time, they are less liable to attack than are wines of lower strengths.

Other things being equal, the higher the alcohol content the less liable the wine is to bacterial attack, but it is not possible to determine exactly the upper limit of the tolerance towards alcohol of the bacteria. In the laboratory, differences have been noticed between the amounts of alcohol which the different types of bacteria are able to tolerate. Thus all strains of Type I examined grew in wine containing 35 per cent. of proof spirit, and a few grew in 37 per cent., but none were able to grow in 38 per cent. under the particular experimental conditions. On the other hand, only a few strains of the other types were able to grow in 35 per cent. of proof spirit and many of them failed to grow in 32 per cent. Wines which are fortified to a strength of 35 per cent. of proof spirit are therefore liable to be attacked by fewer types of bacteria than less highly fortified wines. This point is important, for many strains of Types III, IV, and V are less fastidious in other ways than are the highly alcohol tolerant bacteria of Type I and consequently are able to grow in some wines of low alcohol content which do not support the growth of Type I. Thus some wines which are atacked while containing 32 or 33 per cent. of proof spirit remain sound if fortified to a strength of 35 per cent.

7. Sulphur Dioxide (SO_2)

Sulphur dioxide is the only antiseptic other than alcohol which is commonly added both to musts and to wines which

are intended for human consumption. Its value for this purpose is largely due to the fact that it is not poisonous to man in the concentrations normally used, and moreover, the true wine yeasts are less sensitive to it than most of the undesirable micro-organisms. The oldest method of adding sulphur dioxide consisted of racking the must or wine into casks in which sulphur had previously been burned, so that the fumes were absorbed by the liquid. The fumes from burning sulphur are still widely used for disinfecting casks and wine vats, but other forms of sulphur dioxide are now generally preferred for treating musts and wines because the amounts added can be more accurately controlled.

At ordinary temperatures and pressures sulphur dioxide is a gas, but like many other gases it can be liquefied by high pressure and is obtainable in this form in heavy-walled steel cylinders. This liquefied product may be added directly to the must or wine by means of a special measuring device (sulphitometer) or it may be dissolved in cold water to form a solution of sulphurous acid which is then added to the must or wine. The former method is widely used for treating large quantities of must, but the second method enables the dose to be more accurately controlled and is preferable for wines. The strengths of the sulphurous acid solutions used are commonly between 5 and 6 per cent. and such solutions are obtainable from several of the leading supply houses.

Potassium metabisulphite is also commonly used for treating musts and wines because when added to them it reacts with the acids which are present and liberates sulphur dioxide. In this reaction an equivalent amount of acid in the must or wine is neutralized so that suitable additions of acid should be made when the metabisulphite is added. Potassium metabisulphite contains approximately 57 per cent. of sulphur dioxide, but in practice only about 50 per cent. is available, so that two ounces of the metabisulphite are equivalent to about one ounce of sulphur dioxide.

Strictly speaking, SO_2 is the chemical formula for pure sulphur dioxide, but in oenology the term is used to include not only this substance, but also aqueous solutions of sulphurous acid and the sulphites which occur in musts and wines

which have been treated with sulphur dioxide, sulphurous acid, or potassium metabisulphite. In this publication the term is used in the broader oenological sense.

SO_2 has a very marked antiseptic effect on the lactobacilli which cause spoilage in fortified wines, and although differences in the tolerance of different strains of the bacteria have been noticed, no very highly tolerant strains have been encountered during these investigations. In laboratory experiments none of the strains studied were able to multiply in wine containing 80 parts per million of SO_2 and most strains were completely checked by 75 parts per million, while 100 parts per million actually killed them in a few days. The effectiveness of SO_2 in checking bacterial growth in wine depends not only on the amount added, but also on the uniform distribution of the SO_2 throughout the whole of the wine. Such a uniform distribution is much more difficult to accomplish in a large tank of must or wine than in small laboratory samples, and so bacteria may remain alive in localized zones of low SO_2 concentration even after quite large doses have been added. This point will be referred to again when control measures are being discussed.

When SO_2 is added to must or sweet wine, a portion of it soon enters into combination with the sugars. It is a well-known fact that the SO_2 which is bound in this way is less active than the free SO_2 in preventing the growth of yeasts and it is sometimes supposed that the bound SO_2 is almost without any antiseptic effect. It has been found during the course of these investigations, however, that the bound SO_2 has a pronounced antiseptic effect on the spoilage bacteria and prevents their growth even when very little free SO_2 is present.

It has been pointed out previously that the temperature and acidity of the must and of the wine during the primary fermentation affect the autolysis of yeast cells in the gross lees. Thus temperature and acidity have an important effect on the susceptibility of the wine in addition to their more direct effects on the growth of the spoilage bacteria. No such indirect effect could be demonstrated in the case of SO_2, however, and it appears that the effectiveness of this substance in protecting a

wine from attack depends on the amount present in the wine at any given time, rather than on the time that it was added. Addition of SO_2 to the must is necessary to prevent the growth of spoilage bacteria in the fermentation tank, but as much of this SO_2 is lost during the fermentation, such an addition does not protect the wine against future bacterial attack. The use of SO_2 in preventing bacterial attack is fully dealt with in a later chapter.

8. Tannin

The tannin which is present in some wines is derived almost entirely from the grapes, while other wines have received substantial additions of commercial oenological tannins extracted from other plants. Tannin is commonly added to musts and to wines to promote clarification or to improve flavour and appearance rather than to protect the wines against bacterial attack. Many different opinions have been expressed concerning the antiseptic properties of tannin, but experiments have shown that the addition of normal amounts (less than 1 grm. per litre) of commercial oenological tannins to fortified wines definitely retards the growth of the spoilage bacteria. The amount of tannin necessary to prevent bacterial growth depends on the strain of bacterium and the purity of the commercial tannin used, but the addition of 1 grm. per litre of 'available' tannin has prevented the growth of bacteria in wines containing 35 per cent. of proof spirit in all cases.

The effect of tannin in checking bacterial growth appears to be due to its effect on the bacteria rather than to the precipitation of essential nutrients or accessory growth substances. Thus the effectiveness of tannin depends upon the amount actually present in the wine rather than the amount which has been used in previous treatment of the wine. Tannin is removed from the wine in fining with gelatine, and if excessive amounts of gelatine are used the wine is 'over fined.' Some wines are rendered susceptible to bacterial attack by 'over fining,' but this is due to removal of the tannin, for gelatine in itself has not been found to promote bacterial growth in fortified wines.

Natural grape tannin inhibits bacterial growth to some extent, but is less effective in this respect than the commercial

oenological tannins. This is one reason why wines made from red pressings are attacked by bacteria more frequently than might be supposed in view of their high tannin content. Other reasons for the frequent spoilage of such wines are:

(a) The pH of pressings is usually higher than that of the free run juice and consequently wines made from pressings frequently have a high pH.

(b) The tannin content of pressings may not be as high as is commonly supposed, for some of the material determined as tannin in these wines by usual methods of analysis is colouring matter rather than tannin.

(c) Owing to the aeration to which the skins are subjected during pressing such wines usually contain more yeast cells in suspension than the free run juice, and if conditions become favourable for yeast autolysis, and, as previously pointed out, high pH is one such condition, pressings are rendered more susceptible to bacterial attack.

CHAPTER IV

CONTROL OF BACTERIAL SPOILAGE

WINES which have undergone a considerable amount of bacterial spoilage cannot be treated satisfactorily, and for this reason the aim of control measures should be the *prevention* of spoilage. The possible methods of preventing bacterial spoilage of wines may be conveniently discussed under the following three headings:

1. Prevention of bacterial contamination of the wine.
2. Destruction or removal of the bacteria which have gained access to the wine.
3. Maintenance of conditions which are unfavourable for bacterial growth.

These different types of control measures should not be regarded as alternatives, however, but should be employed in conjunction with one another.

1. PREVENTION OF BACTERIAL CONTAMINATION OF THE WINE

It is practically impossible to avoid bacterial contamination in the winery completely, but it is possible to restrict contamination considerably by general cleanliness and the use of disinfectants. In practice it is highly desirable that the amount of contamination be reduced to a minimum, for the greater the number of bacteria which find their way into a wine, the more likely it is that some of them will multiply and the more difficult it will be to prevent spoilage by employing other methods of control.

With regard to winery hygiene, it may be pointed out that cleanliness should be practised both outside and inside the winery. Heaps of decomposing marc and pools of 'still-wash' attract vinegar flies and provide a 'breeding ground' for many types of micro-organisms including lactic acid bacteria and

vinegar bacteria. If such waste materials are allowed to accumulate and remain close to the winery, the likelihood of wines becoming infected is greatly increased.

The winery itself should receive special attention and the casks, tanks, and various pieces of winery equipment such as hoses, pumps, filters, presses, etc., should be thoroughly cleaned and disinfected after use. Floors and drains should be so constructed as to simplify washing down, and any spilled wine should be washed away and not absorbed with sand or sawdust or allowed to soak into the floor.

Casks and wine tanks when emptied should be thoroughly washed out with water and then well drained. If they have contained unsound wine or if they are to remain empty for some time, they should be disinfected after first being thoroughly cleaned. Tartrate deposits and 'wooden blisters' should be removed from the inside of casks by chipping and scraping; a solution of washing soda in hot water (one ounce per gallon) is of great assistance in loosening tartrate deposits, particularly in small casks. In the case of cement tanks, it is necessary to remove old paraffin wax by melting and scraping it off, and the inside of the tank should then be inspected for crevices and loose surface flakes and repaired if necessary.

Cement tanks may be disinfected by washing down with a solution of sodium hypochlorite containing 250 parts per million of available chlorine. They may also be disinfected by fumigation with chlorine gas in the following way: In a large earthenware pot in the bottom of the tank are placed 2 lb. of bleaching powder and $1\frac{1}{2}$ lb. sulphuric acid for every 1,000 cubic feet of space. The tank is then closed except for a small vent hole. For maximum effect the walls of the tank should be damp at the time of fumigation. If this method of disinfection is used, it must be remembered that chlorine is a very poisonous gas and no one should enter the tank until the chlorine has been removed by ventilation and rinsing. Whichever method of disinfection is used, the tank should be thoroughly rinsed with *clean* water to remove the odour of chlorine before being reparaffined.

One of the commonest methods of disinfecting casks is by

burning sulphur wicks or tablets in them (one ounce of sulphur per 100 gallons capacity). The inside of the cask should be damp when sulphured in order to get the full effect of the treatment, but the cask should not contain free water and care should be taken to see that no molten sulphur falls into the damp cask, or there will be danger of sulphuretted hydrogen (H_2S) being formed and imparting a foul odour to the cask. After being sulphured the cask should be kept closed and the treatment should be repeated about once a month until the cask is dry, if it is to be left empty.

Casks may also be disinfected and kept in good condition when not in use by a solution of potassium metabisulphite. Small casks may be kept filled with a solution of one ounce of metabisulphite and one ounce of sulphuric acid in every ten gallons of water, but a more convenient and cheaper method for hogsheads and puncheons is to use a smaller quantity of a stronger solution. In each hogshead or puncheon are placed two gallons of water, two ounces of metabisulphite, and two ounces of sulphuric acid. The casks are kept standing on end and are turned upside down about every two weeks. This solution will keep casks sound for six months, but it is advisable to add an additional ounce of metabisulphite to each cask at the end of the first three months.

Hypochlorite is also used for disinfecting casks and is very effective for this purpose, particularly if the casks have contained badly infected wines. Casks which are in good condition and have contained only sound wines may be rinsed out with a solution of sodium hypochlorite containing 250 parts per million of available chlorine, but casks which have contained infected wines should be washed out several times with a stronger solution (1,000 parts per million of chlorine) or they may be filled with a dilute solution (150 parts per million of chlorine) for one or two days. Large wooden storage vats may also be fumigated with chlorine gas as directed for cement tanks. Owing to the way in which the chlorine odour clings to wooden casks, it is necessary to wash them out very thoroughly after disinfecting with hypochlorite solutions or chlorine gas. A final rinse with the metabisulphite solution mentioned pre-

viously is of great assistance in removing the last traces of chlorine.

Another useful wash for disinfecting casks is a one per cent. solution of formalin. Casks may also be filled with this solution, and the solution pumped from one cask to another at intervals to keep them wet and sound. Afterwards the casks must be rinsed very thoroughly, however, to remove the odour of formalin before they are filled with wine. A small quantity of ammonia followed by further rinsing with clean water is useful for banishing the last traces of formalin odour.

Hoses, pumps, and other winery equipment may be a very prolific source of bacterial infection, particularly after they have been used for moving spoiled wines. Hoses should be thoroughly washed out with water followed by a solution of hypochlorite (500 parts per million of available chlorine) after use, and should then be rinsed with clean water and placed on sloping racks to drain. Other pieces of winery equipment such as pumps, presses, and filters should be dismantled sufficiently to allow them to be cleaned thoroughly after use. They should then be washed thoroughly with water followed by the hypochlorite solution, and finally rinsed and drained. For disinfecting floors and drains a stronger hypochlorite solution (1,000 parts per million of available chlorine) may be used, but as in the case of the winery equipment, disinfecting should follow washing and does not take the place of washing.

It is particularly important that casks and equipment should be washed and disinfected after having been in contact with infected wines, but this is not the only occasion when such treatment is necessary. It should be remembered that small quantities of even sound wines or lees, if allowed to remain on floors, in drains and sumps, in casks or in hoses, filters, etc., will soon lose portion of their alcohol by evaporation and become a good 'breeding ground' for spoilage bacteria.

As has been previously pointed out, it is highly desirable that bacterial contamination be reduced to a minimum, but in spite of all precautions, contamination cannot be avoided entirely. The bacteria are widely distributed, and therefore spoilage cannot always be prevented by methods such as those already discussed.

2. Destruction or Removal of Bacteria which have Gained Access to the Wine

When bacteria are found to be developing actively in a wine, some rapid means of arresting their development is necessary. If the treatment is to be of value, however, it is essential that it should be carried out before the composition of the wine has been altered appreciably by the bacteria. It is necessary therefore that the bacteria be discovered at an early stage in their development. This, in turn, involves a microscopical examination of all wines and lees at frequent intervals, particularly during the first two years of storage, when they should be examined at least every three months. If bacteria are present, they can usually be detected by a microscopical examination of the lees or, in some cases, by an ordinary inspection of the wine, before they have brought about any appreciable changes in the composition of the wine. If a sample of infected wine in a clear glass or bottle is shaken with a rotary motion while held in front of a light, the characteristic silky, wavy appearance can often be detected before the composition of the wine has been appreciably altered. Wines which are cloudy should be subjected to a very careful examination under the microscope, but if a wine is perfectly clear and bright it does not necessarily follow that no bacteria are present in the lees. The bacteria may grow slowly in the lees for months before the 'condition' of the wine is appreciably affected. Thus all lees should be examined regularly even though the wines themselves appear to be in satisfactory condition.

If bacteria are found in a wine under Australian conditions, they may be assumed to be spoilage bacteria. Reference has been made to certain improvements in the flavour of some European wines as a result of bacterial activity, but there is no evidence of improvement in Australian wines from such a cause. At the present stage of our knowledge the presence of any bacteria in our wines must be considered undesirable.

The bacteria, when detected, may be destroyed by pasteurizing the wine, or they may be removed from the wine by means of a germ-proof filter, or by fining the wine after checking bacterial growth with SO_2.

In laboratory tests none of the strains of bacteria tested survived a temperature of 70° C. (158° F.) for one minute in wine containing 28 per cent. of proof spirit and having a pH value of 4·2. The application of such treatment to fortified wines should therefore kill any spoilage bacteria present. In practice, however, the efficiency of pasteurization depends not only on the temperature to which the wine is heated, but also on the design of the pasteurizer and the care of the operator. It is essential that *all* of the wine which passes through the pasteurizer should be held at 158°–160° F. for at least one minute, and also that recontamination, as a result of leakage between incoming and outgoing wine or other cause, should be avoided.

Bacteria may be removed from infected wine by filtration. Seitz E.K. filter discs and also Ford's Sterilmats grade G.S. have proved quite effective for this purpose in laboratory tests, and have also given good results in commercial practice.

Pasteurization or filtration, if properly carried out, can be relied on to destroy or remove the bacteria which are in the wine at the time of treatment, but they do not preclude the possibility of further trouble resulting from re-infection at a later date. Occasionally, instances of the apparent ability of spoilage bacteria to pass through germ-proof filters or to withstand high pasteurization temperatures are reported by wine-makers. There seems little doubt, however, that such instances are due to faulty technique or equipment or to re-infection of the treated wine. It has been shown that the bacteria are widely distributed, and thus the possibility of pasteurized or filtered wines becoming re-infected must be considered a very real danger unless the treated wines are immediately bottled in sterile bottles, or unless steps are taken to render them unfavourable for bacterial growth. It is possible to pasteurize wine in bottles at 140° F. for 20 minutes, but in order to minimize condition troubles it is usually necessary that the wine be previously bulk pasteurized, cooled, and filtered. Wine may also be bottled in sterile bottles direct from a germ-proof filter and the risk of re-infection thus avoided. Quite often, however, wine has to be pasteurized or filtered some time before it is ready to be bottled. In such cases the filtered or pasteurized wine should be run directly

into casks or tanks which have been thoroughly disinfected (see previous section) and the SO_2 content of the wine raised to 100 parts per million. It is also desirable to increase the acidity and the tannin content of the wine, but no general figures can be given concerning the amounts of acid and tannin which should be added to finished wines, and the winemaker must be guided by his palate in making such additions.

When no pasteurizer or germ-proof filter is available and bacteria are developing actively in a wine, they may be checked by the addition of SO_2. It has been found in laboratory experiments that the addition of 100 parts per million of SO_2 to a wine not only prevents bacterial growth, but actually kills the bacteria present in the wine. Such a result cannot be relied on under industrial conditions, however. Bacteria have been isolated from gross lees after the original musts had been treated with metabisulphite at the rate of 8 ounces per 100 gallons (equivalent to about 250 parts per million of SO_2). The bacteria had not caused spoilage in these cases, and it is probable that their survival was due to the presence of localized zones of low SO_2 concentration as a result of uneven distribution of the metabisulphite in the must. It is likely, however, that the bacteria in such zones would re-infect the whole of the contents of the vat or tank when the SO_2 content of the wine had fallen to a sufficiently low level. It is advisable, therefore, that when SO_2 is added to fortified wine for the purpose of checking the growth of actively growing bacteria, sufficient should be added to raise the SO_2 content to 150 parts per million, and it should be thoroughly mixed throughout the wine. The wine should then be freed from bacteria by filtration or fining and racking.

3. Maintenance of Conditions which are Unfavourable for Bacterial Growth

Bacterial spoilage of wine depends on the two following sets of circumstances:

(a) Bacterial contamination of the wine.
(b) Favourable conditions for the development of the bacteria.

It has already been pointed out that some bacterial contamination is very likely to occur in spite of all ordinary precautions and so the importance of maintaining conditions which are unfavourable for the development of the bacteria becomes fairly obvious.

The effects of various factors on the growth of the spoilage bacteria in wines have been determined experimentally and were discussed in Chapter III, and this information serves as a guide in determining how spoilage may be prevented by maintaining conditions which are unfavourable for bacterial growth.

Reference has already been made to the necessity for modifying the composition of wines, which have been freed from living bacteria by pasteurizing or filtering, in order to prevent the growth of any bacteria which may find their way into the wine later. Such modifications are usually effected by adding SO_2 and tannin and increasing the acidity within the limits of palatability. The extent to which a wine can be modified at this stage without losing in quality is very limited, however, and the addition of acids and tannin to a finished wine, as a means of checking bacterial growth, can only be regarded as a belated attempt to correct faults due to the composition of the must and to the conditions under which the wine was made. Far better results are obtained if control of the various factors which affect bacterial growth is commenced *before the primary fermentation.*

(1) Control of Acidity

It has been shown that the susceptibility of wine to bacterial spoilage is very largely governed by the acidity of the must and of the resulting wine, and thus control of acidity should play an important part in control of spoilage.

As already explained in Chapter III, Section I, the acidity of must or wine may be expressed in two ways:

(a) As titratable acidity, i.e., total amount of acid present, usually expressed as grms. of tartaric acid per litre.

(b) As the concentration of free hydrogen ions, i.e. the amount of acid which has undergone dissociation, expressed as pH values.

The pH value undoubtedly provides the more reliable guide to the influence of acidity on bacterial growth and on the autolysis of yeast cells, and in practice it is the wines with the highest pH value (lowest acidity) which are most readily attacked by bacteria. On the other hand, contrary to the opinion of some oenologists, sourness or acidity as registered by the human palate seems to be more closely related to the titratable acidity than to the pH value of the wine.

Thus there are several points to be considered in connection with the control of acidity.

(a) It is the pH value rather than the titratable acidity of must and wine which indicates susceptibility to bacterial spoilage. Nevertheless, although it is desirable that the pH value should be below 3·6, acidity should not be adjusted entirely on a pH basis without taking into account the titratable acidity and sourness of the finished wine.

(b) Although the titratable acidity gives a better indication of sourness than does the pH value of a wine, it is not possible to give a standard titratable acidity value which would represent the greatest amount of acid which could safely be added to any sweet wine. Sourness depends not only on the amount of acid present, but also on the extent to which the acid taste is masked by other substances such as sugar. Some wines become too sour when the titratable acidity is raised to 4·5 grms. per litre (as tartaric acid), but most sweet wines are improved by raising this amount somewhat, and some may contain considerably larger amounts and still be palatable. For this reason the winemaker must rely very much on his palate and his experience in deciding the amount of acid which can safely be added.

(c) It is better to add acid to the must than to the wine, for a low pH (high acidity) in the fermentation tank and during the period that the wine is on the gross lees, inhibits the autolysis of yeast cells and the accompanying liberation of accessory growth substances which stimulate bacterial growth. Addition of acid to the must rather than to the wine also results in a better flavoured wine. Moreover, fermentation tends to proceed more steadily in the acidified must than in must of low acidity, and consequently control of fermentation-temperature

is facilitated; this last point is particularly important in warm districts, which are the very districts where the musts are most often deficient in acidity.

(d) When adjusting acidity the aim should be to lower the pH value of the must and of the resulting wine without unduly increasing the titratable acidity and sourness of the wine. For this reason tartaric acid is better than citric acid for acidifying the must. Smaller quantities of tartaric acid than of citric acid are required to lower the pH of a must to the desired value, and thus, if the acidity is adjusted on a pH basis, the titratable acidity of the must acidified with tartaric acid will be less than if citric acid were used. Although portion of the tartaric acid is precipitated in the form of potassium bitartrate (cream of tartar) during fermentation and following fortification, this precipitation is an additional point in favour of tartaric acid, for it lowers the titratable acidity and decreases the sourness of the wine considerably more than it raises the pH value.

The actual amount of tartaric acid to be added will, of course, vary with different musts, and will depend on such factors as variety of grape, stage of ripeness, climate, and soil. It has been found that fortified wines made from musts with pH values below 3·6 are much less susceptible to bacterial attack than are wines made from musts with higher pH values, and ideally, the pH of all musts should be below 3·6 at the commencement of fermentation. Unfortunately, however, the pH values of some of our musts are as high as 4·3, and the cost of adjusting the pH to below 3·6 by the addition of tartaric acid alone, often renders such a practice quite uneconomic. To a large extent such very high pH values can be avoided, however, by crushing the grapes before they become over-ripe and by blending musts of different degrees of ripeness. It is known that the pH value of grape juice gradually rises during the ripening of the grape, and it has been noticed in the course of these investigations that many of the musts with abnormally high pH values come from grapes which do not usually yield juice with a high density, but which have been left on the vines till late in the season in order to obtain high Baumé readings. This practice is probably justifiable if the must is to be used for the production of distillation wines,

but the winemaker should realize that he is sacrificing acidity for the sake of the extra sugar, and wines made from such musts are likely to be very susceptible to bacterial spoilage.

Pressings call for special mention, for the pH of such material, particularly of the final pressings, is usually higher than that of the 'free-run' juice. It has been noticed that wines which have been made from pressings are frequently attacked by bacteria in spite of their high tannin content, and there seems little doubt that this is largely due to the high pH value of such material.

There are, then, three ways in which the winemaker can control acidity. The ideal method of controlling acidity is to harvest and crush the grapes before the pH value has risen above about 3·6. It is realized, however, that such a course is often impracticable with certain varieties and in certain districts, and so the next best way is to lower the pH value (increase the acidity) of the must by the addition of tartaric acid.

The third and least desirable method of controlling acidity is to add acid to the wine after it is made. Tartaric acid may be added to young wines, but if the wines are more mature the precipitation of potassium bitartrate which follows the addition of tartaric acid may be a serious disadvantage as it necessitates an additional period of storage to allow the wine to become sufficiently stable for bottling. For this reason citric acid is often preferable to tartaric acid for adjusting the acidity of the *finished wine*, but the results are less satisfactory than when tartaric acid is added to the *must*. Very often when acid is added to finished wines which have a very high pH, it is found that the wines become unpleasantly acid in flavour long before the pH has been lowered to 3·6. The only course open in such cases is to increase the acidity as much as possible within the limits of palatability, and to be particularly careful in applying the other methods of control discussed in this chapter.

(2) Control of Temperature

The popular belief that bacterial spoilage occurs only when wines are held at temperatures in the neighbourhood of 95° F. (35° C.) is not supported by experimental results. It is true

that the growth of some strains of the bacteria in musts and in wines of low alcohol content is accelerated by such high temperatures, but temperatures above 77° F. (25° C.) have been found to retard rather than accelerate bacterial growth in fortified wines. Although the bacteria grow more rapidly in fortified wines, at temperatures between 68° and 77° F. (20° and 25° C.) than at lower temperatures, the amount of spoilage caused by bacterial growth at temperatures as low as 59° F. (15° C.) may be quite considerable over a long period. Thus, the bacteria grow quite actively over the range of temperatures at which wine is normally stored in Australian wineries, and control of storage temperature does not appear to offer much promise as a direct means of checking bacterial growth.

On the other hand, the temperature at which the primary fermentation is carried out, and at which the wine is stored prior to the first racking, may have a very great influence on the susceptibility of the wine to bacterial spoilage. Most winemakers realize that high fermentation-temperatures are dangerous during the making of dry wines, since they frequently lead to 'stuck' fermentations and the resulting wines are notoriously prone to bacterial attack. It has been shown, however, that high temperatures favour the autolysis of yeast cells in the gross lees and so have an important effect on the susceptibility of the wine even when the fermentation does not 'stick.' No fixed temperature can be given as marking the boundary between safety and danger under all circumstances, but, within the range of temperatures at which fermentations are normally carried out (approximately 70° to 95° F.), the higher the temperature the greater the likelihood of yeast cells undergoing autolysis rapidly. Temperatures of 90° F. or more are definitely dangerous.

The temperature reached by the fermenting must depends on the initial temperature of the grapes when crushed, the amount of heat generated during fermentation, and the amount of heat lost by radiation and removed by 'cooling.' Thus the amount of cooling required can be reduced by crushing the grapes when they are cool (as far as possible) and by the use of fermentation tanks with thin walls and of moderate size which thus lose heat rapidly. The addition of suitable

doses of SO_2 to the must and the use of a 'steady' rather than a 'tempestuous' fermenting strain of yeast as a starter, also reduce the amount of cooling necessary, for the more slowly the fermentation proceeds the smaller the accumulation of heat and rise in temperature.

Of the various methods used for cooling the fermenting must, one of the most efficient is running the must through a tubular heat exchanger in which it is cooled by cold water or cold brine. In this country the must is more commonly cooled by immersing cooling coils or pipes in the fermentation tanks and running cold water through them. This second method also gives satisfactory results, particularly if the fermentation tanks are small. Whichever method is used, however, it is important that the temperature of the fermenting must should be determined frequently and that the must be cooled adequately as soon as the temperature rises above 85° F. High temperatures during the early stages of fermentation are particularly undesirable, since they lead to a very rapid tempestuous fermentation accompanied by a sharp rise in temperature which is difficult to control.

Experimental results have shown that high temperatures are dangerous not only during fermentation, but up till the time of the first racking. For this reason, newly fortified wines should not be stored in positions where they are exposed to high temperatures.

(3) Time of Racking

The accessory growth substances which are liberated from autolysing yeast cells appear to play a very important part in rendering some wines susceptible to bacterial spoilage. It has already been pointed out that autolysis of yeast cells occurs much more rapidly in some wines than in others, and for this reason no set time of racking can be recommended as safe for all wines. It seems logical, however, to curtail as much as possible the period during which the wine is in contact with autolysing yeast cells, and the winemaker should aim at having the young wines clear and racked off the gross lees as early as possible. In this connection, the use of pure yeasts in conjunction with SO_2 for carrying out the primary fermentation, and

the control of fermentation-temperatures are highly desirable since the resulting wines usually clear earlier and more thoroughly than do wines made by uncontrolled fermentation. Strains of yeasts differ considerably from one another in the rate at which they sink to the bottom of the wine after fermentation. Wines which have been fermented by the so-called agglomerating yeasts clear more rapidly, and so can be racked earlier than wines fermented by other strains.

(4) Use of SO_2

SO_2 is commonly added to wines both to check the growth of bacteria already present and also to protect the wine from subsequent bacterial attack. In the laboratory it has been found that the spoilage bacteria fail to grow in fortified wines which contain between 75 and 80 parts per million of SO_2 even when the amount of *free* SO_2 is only very small. In practice SO_2 is usually added to the must in amounts which range from 100 to 250 parts per million—equivalent to 1⅗ to 4 ounces of liquefied sulphur dioxide, 3⅕ to 8 ounces of potassium metabisulphite or 1⅗ to 4 pints of a five per cent. solution of sulphurous acid to every 100 gallons of must. The smaller dose is used with clean, sound, ripe grapes in cool weather and heavier doses are used with damaged or mouldy grapes and in hot weather. These doses of SO_2 have been prescribed chiefly for the purpose of preventing the growth of moulds and various undesirable types of yeasts present on the grapes, and they are also quite effective in preventing the growth of bacteria in the fermenting must. The addition of SO_2 to the must is important, for it has been shown that some of the spoilage bacteria grow very readily in fermenting must if no SO_2 is used. In such cases, the wines may be already spoilt before fortification. Another important advantage of adding SO_2 to the must is that fermentation proceeds more steadily in the sulphited must and consequently the temperature of the fermenting must is more easily controlled.

A considerable portion of the SO_2 which is added to the must is lost during the fermentation. In laboratory experiments this loss was of the order of 50 per cent. of the SO_2 added. Biolletti and Cruess (1912) found this loss to vary

from 14 to 60 per cent. of the amount of SO_2 added. The SO_2 which remains in the wine after fortification is almost entirely 'bound SO_2,' but provided it is sufficient in amount, it is quite effective in preventing bacterial growth. Because of the loss of SO_2 referred to above, however, it is advisable to determine the SO_2 content of the wine immediately after racking and, if necessary, make further additions.

The amount of SO_2 which a fortified wine should contain depends on several factors. In laboratory experiments determinations have been made of the amount of SO_2 needed to check bacterial growth in fortified wines when all other conditions were favourable for growth. In practice, however, it is the duty of the winemaker to ensure that all other conditions are *not* favourable for bacterial growth, and consequently smaller amounts of SO_2 may be effective in many wines. There is, however, another very important difference between laboratory experiments and industrial conditions, for although it is comparatively easy to mix the added SO_2 uniformly throughout a small laboratory sample, a similar uniform distribution throughout the contents of a large vat or tank is much more difficult to accomplish. Thus, localized zones of low SO_2 concentration in which the bacteria are able to develop may occur in large storage vessels. In view of these factors, it is recommended that the amount of SO_2 in fortified wines should be approximately 100 parts per million after the first racking, but this amount may be increased to 120 or even 150 parts per million in wines which are particularly prone to bacterial attack because of high pH. The dose of SO_2 needed to destroy bacteria which are actively growing in wine has already been discussed in another section of this chapter. During storage, particularly in small casks, the SO_2 content of the wine falls on account of evaporation and oxidation, and this loss is increased by the aeration which accompanies such operations as racking. Under these circumstances, it is advisable that the SO_2 content of the wine should be determined from time to time and the necessary adjustments made. Quinn (1940) finds that the loss of SO_2 from wines stored in hogsheads is about 100 parts per million in twelve months and ad-

vocates that SO_2 determinations and adjustments should be made at intervals of three to six months.

SO_2 retards oxidation of wine and is sometimes added to wines which tend to become 'flat' owing to over-oxidation, in order to 'freshen them up,' but it must be borne in mind that high concentrations of SO_2 retard the normal aging of wine, which is largely oxidative in character. For this reason it is not advisable to maintain a high level of SO_2 in wine continuously and rely on this as the sole means of preventing bacterial spoilage. SO_2 is a very necessary adjunct to the primary fermentation, and a concentration of over 100 parts per million is permissible in a young wine, but if the wine has been made by sound methods with due regard to control of acidity and the other factors mentioned in this chapter, it should be possible to allow the SO_2 content to fall gradually to 60 parts per million without risk of bacterial spoilage.

(5) Fortification

Australian sweet wines are commonly fortified to a strength of 35 per cent. of proof spirit, but in some cases the alcoholic strength is not raised above 32 to 34 per cent. of proof spirit. Wines which contain 35 per cent. of proof spirit are by no means immune from bacterial attack, but usually they are less susceptible than the wines which have been less highly fortified. Most strains of the spoilage bacteria of Types III, IV, and V have a lower tolerance of alcohol than have those of Type I, but they are less fastidious in some other respects and sometimes grow quite vigorously in wines which do not support the growth of Type I. Thus some wines which undergo bacterial spoilage when containing only 32 to 34 per cent. of proof spirit may remain sound if fortified to a strength of 35 per cent. of proof spirit.

When a wine is fortified, it is important that the fortifying spirit should be thoroughly mixed with the wine. Unless special attention is devoted to this operation, the wine near the bottom of the container may have a much lower alcohol content than the wine near the top. In such cases bacteria may grow very rapidly in the bottom of a tank of wine which nominally has been quite highly fortified.

(6) Tannin Content of Wines

In laboratory experiments it has been found that bacterial growth in fortified wines is definitely retarded by the addition of 0·5 grm. of tannin per litre (8 ounces per 100 gallons), and is completely checked by the addition of 1 grm. of tannin per litre (1 pound per 100 gallons). Tannin is commonly added to musts and to wines to facilitate fining with gelatin subsequently, and also to improve the flavour and protect the colour of certain wines which contain insufficient tannin. It has a very marked effect on the flavour of wine, however, and the amount which can be added without adversely affecting the wine seldom exceeds 0·5 grm. per litre, and is often far below this figure. It is unlikely, therefore, that many wines can be protected from bacterial spoilage by additions of tannin alone. However, the effect of tannin on the spoilage bacteria provides an additional reason for its use within the limits imposed by considerations of palatability.

It has been shown experimentally that wines may become susceptible to bacterial spoilage as a result of over-fining with gelatin. This effect seems to be due simply to the removal of tannin, for gelatin in itself did not increase bacterial growth in wines. It is important therefore, that appropriate amounts of tannin should be added to wines which are to be fined with gelatin, or, alternatively, bentonite may be used as a fining agent instead of gelatin.

Conclusion

As a result of experimental investigation it has been shown that a number of factors play an important part in favouring or retarding bacterial development. It appears that the most important factors to be considered in this regard are as follows:

(a) The acidity of the must.

(b) The care of the primary fermentation, including the use of SO_2 and pure yeasts and control of temperatures.

(c) The care of the wine during storage, including fortification, control of temperature prior to the first racking, thorough clarification and early racking, the use of SO_2, and the use of tannin and avoidance of over-fining with gelatin.

It is perhaps natural that many winemakers have their own 'pet theories' regarding the control of spoilage. Some are enthusiastic about pasteurizing, others about germ-proof filters, and still others about SO_2, acidity or tannin. The various control measures which have been discussed should not be regarded as alternatives, however, for in most instances they are complementary.

It is true that the growth of bacteria can often be prevented by controlling only one of the factors mentioned above. Their development can be checked quite effectively by the addition of acid, alcohol, SO_2, or tannin, provided that the amount added is sufficient. In practice, however, it is often found that the amount required to check bacterial growth completely would render the wine quite unpalatable. As regards many factors, there is a rather narrow margin between the level at which the development of the organisms is noticeably retarded and the level at which the palatability of the wine is adversely affected. It should be the aim of the winemaker to keep not one, but *all* factors adjusted within this margin. It cannot be too strongly emphasized that the winemaker should ensure that as many factors as possible are unfavourable for bacterial growth. He will almost certainly meet with disapointing results if he attempts to prevent bacterial spoilage by control of only one factor.

PART II

CHAPTER I

METHODS OF ANALYSIS

EXCEPT where otherwise intimated, the following methods of analysis have been used throughout these investigations:

Alcohol, total titratable acidity, volatile acidity, total SO_2, free SO_2, and tannin: A.O.A.C. official methods (1935).

Lactic acid: Roettgen's modification of Möslinger's method (Leach, 1920).

Reducing sugars: Lane and Eynon's method (1934).

pH: Quinhydrone electrode.

Identification of volatile acid: The distillate was neutralized with barium hydrate, evaporated to dryness, and the percentage of barium in the barium salt was determined.

Identification of type of lactic acid: Lactic acid was separated by ether extraction, neutralized with zinc carbonate, and the water of crystallization and percentage of zinc in the zinc salt were determined.

Qualitative test for mannitol: A small portion of the culture or wine was evaporated to a thick syrup and this residue was washed with alcohol. Mannitol, if present, separated out as characteristic rosettes of fine, needle-like crystals.

CHAPTER II

THE NATURE AND CAUSE OF THE SPOILAGE OCCURRING IN FORTIFIED WINES

THE nature of the spoilage which occurs in fortified wines has been investigated by:

(1) Collecting and examining a large number of samples of spoiled wines and comparing these with sound wines of similar types.

(2) Isolating bacteria from wines and lees and studying the characteristics of these bacteria.

(3) Inoculating sterile sound wines with pure cultures of bacteria and determining the changes brought about by the bacteria under controlled conditions.

1. THE CHARACTERISTICS OF SPOILED WINES

The appearance, flavour, and odour of spoiled wines have already been discussed in Part I.

The results obtained by analysing sound and spoiled fortified wines indicate that the spoiled wines have a higher volatile acidity and often a higher fixed acidity than sound wines of similar type. Actually the fixed acidity of the wine always increases during bacterial spoilage, but as the fixed acidity of wines which undergo bacterial spoilage is often very low at the time of infection, the spoiled wines are not always characterized by an abnormally high fixed acidity.

Although it is usually found that wines with a high pH are more readily attacked by bacteria than wines with a low pH, those wines which have undergone a considerable amount of spoilage often have a pH lower than that of sound wines of similar type. It will be shown in a later section that the pH of a wine falls as a result of bacterial spoilage.

The figures presented in Table 1 represent the results of analyses carried out on samples of wines which were submitted

TABLE 1

Analyses of Sound and Spoiled Wines.

Wine No.	Type of Wine.	Remarks.	Alcohol vol. %.	pH.	Volatile acidity g/l acteic acid.	Fixed acidity g/l tartaric.	Reducing sugars dextrose %.
55	Fortified dry white	Sound	19·0	3·75	0·56	3·83	0·96
56	" " "	Badly infected	19·0	3·52	1·10	5·92	0·29
26	Sweet white	Sound	19·5	3·80	0·40	3·52	9·8
25	" "	Infected	19·5	3·80	0·90	3·93	7·2
34	" "	Slightly infected	19·8	4·00	0·62	3·40	Not determined
8	Sweet red	Sound	19·9	3·80	0·60	3·85	6·3
7	" "	Very badly infected	19·7	3·50	3·10	5·30	Not determined
9	" "	" " "	20·0	3·75	2·10	6·50	" "

for examination, and they are typical of many such results. Details of the changes brought about by the bacteria when grown in wine under controlled conditions are presented in a later section.

The volatile acid in spoiled wines was found to be acetic acid and the wines also contained appreciable amounts of lactic acid, while the presence of mannitol was demonstrated in some but not all of these wines. Carbon dioxide is also formed during the bacterial spoilage of fortified wines, and some wines which had undergone spoilage in bottles were found to be heavily charged with this gas.

2. The Bacteria Causing Spoilage of Fortified Wines

(1) Isolation and Culture

(a) *Culture media.* In the early stages of the investigations considerable difficulty was experienced in selecting a suitable culture medium. Most strains of the bacteria failed to grow at all in the usual laboratory media, and although some growth occurred in yeast water, bacto-yeast-extract medium and marmite infusion, these media did not give reliable results. Yeasts and acetic acid bacteria, as well as many common dust- and water-borne contaminants, are present in many samples of lees and spoiled wines. It was therefore considered necessary that the medium used for isolating the spoilage bacteria should be highly selective, i.e., it should so far as possible be suitable for the growth of the spoilage bacteria but unsuitable for the growth of other organisms.

After a number of different culture media had been tested, an autolysed yeast medium was selected and used throughout the investigations. This medium has given very satisfactory results and is prepared as follows:

A one-pound (454 grm.) cake of fresh pressed yeast is divided into four equal portions. Each of these is crumbled into a one-litre Erlenmeyer flask containing 600 c.c. of sterile water in which 1·0 grm. of potassium dihydrogen phosphate and 0·2 grm. of magnesium sulphate are dissolved. A few c.c. of chloroform are added to the yeast suspensions and the flasks are then held at a temperature of 45° C. for ten days in order

to promote autolysis of the yeast cells. During this period the mouths of the flasks are plugged with cotton wool and covered with waxed paper or tinfoil to prevent excessive evaporation. After the period of autolysis the flasks are heated once in the steamer for twenty minutes. (This heating coagulates a certain amount of material which would otherwise be precipitated in the tubes of medium during final sterilization.) The liquid is then filtered perfectly clear on a Buchner funnel, with a Whatman No. 2 filter paper and a filter-aid, such as diatomaceous earth or asbestos pulp, and the filtrate from each flask is made up to one litre with water. At this stage the liquid is light amber in colour and shows a blue-green fluorescence in daylight. It usually contains about 0·2 per cent. of total nitrogen (Kjeldahl), of which approximately a third is in the form of amino nitrogen as determined by the method of van Slyke. For most culture work other than the determination of fermentation reactions, 0·3 per cent. of potassium hydrogen tartrate and 2·0 per cent. of dextrose are added to the autolysed yeast extract. The pH is adjusted to different levels according to the purpose for which the medium is to be used, but medium with a pH of approximately 4·5 has been used for isolations and for most of the other culture work. The medium is dispensed in test-tubes, flasks, or bottles, and is sterilized by heating in the steamer for 20 minutes. Because of the low pH of the medium, a single treatment in the steamer is sufficient. In order to check the growth of yeasts and acetic acid bacteria in initial cultures from wines and lees, 15 per cent. of sterile alcohol is added to the medium immediately before inoculation. For plate cultures, the medium is solidified with agar or gelatin.

(b) *Method of isolation.* Many of the samples of infected wines examined appear to have reached a very advanced stage of spoilage long before being sent to the laboratory. Such samples usually contain immense numbers of bacteria, but as most of these appear to be dead the samples are frequently quite unsuitable as sources from which to make isolations. Wines in which the bacteria had only recently commenced growing and fresh gross lees have generally proved to be the most suitable materials from which to isolate the bacteria.

Isolation was carried out by means of plate cultures which were inoculated from enrichment cultures. The initial enrichment cultures were made by adding small amounts of the infected wines or lees to tubes of autolysed yeast medium to which 15 per cent. of sterile alcohol had previously been added. The amounts of inoculum used varied from a few drops to a few c.c. according to the number of bacteria present in the wines or lees. After being inoculated, the tubes were closed with waxed corks in order to prevent undue loss of alcohol through evaporation, and they were then incubated at 23° C. This combination of low pH and high alcohol content has proved very effective in checking the growth of yeasts, moulds, acetic acid bacteria and various other contaminants which are often present in the samples of wines and lees or on the necks of the bottles containing the samples.

Growth in the initial cultures was usually apparent after a period of one to three weeks, and these cultures were then used to inoculate plate cultures. A small portion of an enrichment culture was suitably diluted with sterile water, and different amounts of this dilute suspension were mixed with 5 c.c. of sterile wine in each of several sterile petri dishes. About 10 c.c. of autolysed yeast agar (5 per cent. agar), which had previously been melted and cooled to 45° C., was then mixed with the inoculated wine in each petri dish. This procedure ensures that the bacteria are distributed throughout the lower portions of the medium where they grow most vigorously, and the presence of wine in the solidified medium has been found to improve the growth of most strains. The plate cultures were incubated at 23° C. and small colonies appeared below the surface of the medium after a period which varied from a few days to several weeks, according to the strain of bacterium. In many cases the colonies appeared only below the surface, but some of the more vigorous strains produced a few surface colonies also. A portion of a selected colony was plated a second time to ensure purity, and the colonies occurring on the second series of plates were used to inoculate tubes of the standard autolysed yeast medium.

A hundred and ten strains of bacteria have been isolated from spoiled wines and lees by the method described, and

these have been grouped according to their behaviour under cultural conditions. Several strains, representing each of the groups thus obtained, have been subjected to further study as regards their effects on wines and their responses to different environmental factors.

(2) Distribution of the Bacteria

Some data concerning the distribution of the bacteria were obtained by collecting samples of wines and lees from a number of wineries. During the 1937 vintage season samples of musts and wines were collected from the fermentation tanks in several wineries, and at the end of the 1940 season 139 samples of gross lees were collected from wineries in most of the main wine-producing districts of Australia. The samples were taken to the laboratory for examination, and although bacteria could be detected in only a few samples by direct microscopical examination, they were cultured from almost every sample.

The bacteria which were cultured from these samples were not all alike, but comprised several different types which are described in detail in the following section. Some types tolerate greater concentrations of alcohol than others, but the medium used for making the cultures contained between 15 and 16 per cent. of alcohol, so that only bacteria able to tolerate this concentration of alcohol were able to grow. It is likely that other strains less tolerant towards alcohol, and thus not capable of multiplying in fortified wines, were also present in many of the samples.

It is worthy of note that most of the wineries from which the samples were obtained are well equipped modern wineries in which disinfectants are used and modern standards of cleanliness observed. In most cases the musts had been treated with sulphurous acid or potassium metabisulphite at the usual rates, but although this treatment had probably prevented bacterial growth, it evidently did not destroy every bacterial cell under industrial conditions.

From the foregoing data it appears that bacteria which are capable of developing in fortified wines are widely distributed,

but develop sufficiently to spoil the wine only if and when conditions become favourable for their growth. The fact that bacterial spoilage is not more common in our fortified wines must therefore be attributed to the general unsuitability of these wines as media for bacterial growth. It seems likely that the spoilage bacteria are present in practically all wineries and that a wine which remains sound does so because it is not susceptible to bacterial attack rather than because it has not been exposed to bacterial infection.

(3) Characteristics of the Bacteria: Results of the Study of 110 Strains

All of the strains are gram-positive, non-motile, non-sporing rods which produce acid and gas from dextrose and levulose. The acid is in part volatile and in part non-volatile, and the gas consists entirely of carbon dioxide. The volatile acid was identified as acetic acid, while the non-volatile acid was identified as optically inactive lactic acid. In addition to these products, mannitol is always present in cultures grown in the presence of levulose.

From the above description it is evident that all of the strains belong to the gas-producing or heterofermentative section of the genus *Lactobacillus*. In order to identify the bacteria more accurately and to compare them with published descriptions of related organisms, a study was made of their morphology, cultural characters, fermentation reactions, optimum temperature range and optimum pH range.

For the determination of fermentation reactions, the sugars and organic acids were dissolved in distilled water, sterilized by means of a Seitz E.K. filter and then added to sterilized autolysed yeast medium which was subsequently tubed aseptically. Two c.c. of a 2 per cent. alcoholic solution of brom cresol green were included in each litre of medium and the pH of the complete medium was approximately 4·7 in each case. The cultures were incubated at 25° C. for four weeks, and in all cases the times taken for growth and acid production to become apparent were noted. The production of acid from the sugars and the decomposition of the organic acids were indicated by the change in colour of the indicator and also by

titration of the cultures. In no case did appreciable growth occur in the autolysed yeast medium to which no sugars or organic acids had been added.

Considerable variation was noted in the vigour with which different strains attacked certain sugars, notably sucrose and maltose, so that it was often very difficult to make any sharp distinction between strains which fermented these sugars and strains which failed to do so. On the other hand, the sugars, lactose, xylose, and arabinose, were in most cases either fermented vigorously or else not fermented at all. Consequently these sugars appear to be of more value than sucrose and maltose for differentiating species and types of the bacteria.

The optimum pH range was determined by growing cultures in autolysed yeast medium containing 2 per cent. of dextrose. The medium was buffered with M/20 phosphate and the pH adjusted with NaOH to the following values: 3·45, 3·95, 4·45, 4·93, 5·40, 5·90, 6·34, and 6·75. The medium was sterilized by filtration and tubed aseptically. Tubes were inoculated with one drop of vigorous six-day-old cultures and were incubated at 25° C. The optimum pH range for growth was determined by inspecting the cultures at frequent intervals and noting the tubes in which the initial growth was most vigorous. It has been shown by Fornachon, Douglas, and Vaughn (1940) that the pH at which cell multiplication is most rapid is approximately the same as the pH at which dextrose is most rapidly fermented, by heterofermentative species of the genus *Lactobacillus*.

Autolysed yeast medium containing 2 per cent. of dextrose and having a pH of 4·7 was used for determining the optimum temperature range. Tubes of this medium were inoculated with one drop of six-day-old cultures and were incubated at different temperatures. The optimum temperature range for growth was determined by inspecting the cultures and also by titrating them after an incubation period which varied from six to ten days according to the strain of organism.

Some idea of the relative alcohol tolerance of different strains was obtained by culturing them in susceptible wine containing 14, 16, 18, 20, 21, and 22 per cent. by volume of alcohol. These cultures were made in 4-ounce bottles fitted

with corks which had previously been heated in molten paraffin wax. After the contents of the bottles had been inoculated and the corks placed in position, the tops of the bottles were dipped in molten 'vaspar' (equal parts paraffin wax and vaseline). The seal thus effected prevented any appreciable loss of alcohol due to evaporation. The bottles were incubated at 23° C. for 12 weeks and examined for bacterial growth from time to time. It is not suggested that the results thus obtained represent the limit of tolerance of the bacteria towards alcohol under industrial conditions. The results do, however, represent the tolerance under the experimental conditions and were determined primarily as an aid to identification rather than to define limiting conditions industrially. It is probable, however, that the results also indicate which strains are able to tolerate the greatest amounts of alcohol under industrial conditions.

On the basis of the observations outlined above, the 110 strains have been divided into five groups or types as follows:

Type I, which is represented by 27 strains, differs appreciably from the other four types both in morphology and in fermentation reactions. Each of the 27 strains has been grown in wine containing 20 per cent. by volume of alcohol, and most of the strains which have been isolated from spoiled wines containing 20 per cent. or more of alcohol are of this type. Type I appears to be the most important organism causing spoilage of wines which contain not less than 20 per cent. of alcohol. The organism is very exacting in its growth requirements and does not grow in grape juice unless autolysed yeast is added. When first isolated it grows very poorly in the absence of alcohol, but grows very well in autolysed yeast medium containing 10 to 15 per cent. of alcohol, and, after being subcultured several times in medium with a low alcohol content, it grows very well in the absence of alcohol. Although some degree of habitation seems to be necessary to enable the organism to grow in the absence of alcohol, no evidence of alcohol utilization has been obtained.

Type I occurs as rods 0·4 to 0·6 microns in width and 2 to 4 microns in length, and also as long thread-like filaments which are often over 50 microns in length. Chains of rods and fila-

ments between 5 and 10 microns in length are common, and predominate in most cultures in fortified wines. After repeated subculture in autolysed yeast medium, chains of short rods become very common. In some spoiled wines the organism occurs as very long tangled filaments and chains of rods which form a flocculent deposit on the bottom of the container. The conditions which favour the filamentous type of growth are not fully understood, but in the laboratory very pronounced filamentous growth has been observed when subcultures are made in a medium containing appreciably more or appreciably less alcohol than the parent culture. A similar phenomenon has been noticed when subcultures are made in a medium the pH of which is considerably higher or lower than that of the parent culture, and also when subcultures are made in a medium containing an appreciable amount of tannin. It appears that filamentous growth is favoured by conditions which the organism is able to tolerate but to which it is not well adapted. No correlation between the age of cultures and the appearance of filamentous forms has been noticed. Some strains show a much greater tendency to grow as filaments than do others, but in all cases the filaments readily give place to short chains and single rods after repeated subculture under optimum conditions. The reverse change, i.e. from short chains and rods to long filaments, does not occur with quite the same degree of regularity, however. The different types of growth are illustrated by the photomicrographs. Fig. 1 shows the type of growth that occurs under optimum conditions, and represents a vigorous six-day-old culture in autolysed yeast medium after repeated subculture in this medium. Fig. 2 represents a six-day-old culture in autolysed yeast medium containing 15 per cent. of alcohol, and is the first subculture in such a medium after a long series of subcultures in alcohol-free medium. Fig. 3 represents a sixteen-day-old culture of the same type as Fig. 2. Figs. 1, 2, and 3 represent the same strain of organism grown under different cultural conditions. With repeated subculture in the medium containing 15 per cent. of alcohol the filaments give place to the type of growth shown in Fig. 1, and in many cases the filaments reappear when the organism is again subcultured in the alcohol-free

medium. The type of growth thus depends not simply on the composition of the medium in which the organism is growing, but rather on the relationship between the composition of this medium and that of the medium to which the organism has become habituated. Somewhat similar variations in morphology were observed by Müller-Thurgau and Osterwalder (1913), who found that *B. mannitopoeum* and *B. gracile* formed long filaments in certain acid fruit juices and other media in which the bacteria grew with difficulty. Pederson (1938) states that weakened strains of lactobacilli and strains grown under unfavourable conditions tend to grow out into long filaments. Shimwell (1935) observed that *Saccharobacillus pastorianus* (syn. *Lactobacillus pastorianus*) occurs both as rods and as long filaments in beer and that the long filaments are favoured by beer with a high alcohol content.

Type I does not grow well on solid media. On agar plates the colonies are of pinhead size, develop only below the surface of the medium, and in some cases appear only after the plates have been incubated for 2 or 3 weeks. Only dextrose and levulose are fermented vigorously, and most strains attack levulose more vigorously than dextrose. Many strains form a little acid from sucrose and maltose. Citric, malic, and tartaric acids are not attacked by any of the strains which have been studied.

Types II, III, IV, and V differ from type I and resemble one another in their morphology and cultural characters. Although variations do occur in both morphology and cultural characters, these organisms cannot be distinguished from one another with any certainty except by their fermentation reactions. Types II, III, IV, and V occur as rods 0·6 to 0·8 microns in width and 2 to 4 microns in length. Chains and filaments also occur, but are much less common than in the case of type I. The similarity between the four bacteria is shown by the photomicrographs. Figs. 4, 5, 6, and 7 represent young vigorous cultures of types II, III, IV, and V respectively in autolysed yeast medium. In general, these organisms grow more rapidly and are rather less exacting in their growth requirements than type I, and many strains grow quite readily in ordinary grape juice if the pH is not too low. Types II,

III, IV, and V do not tolerate such high concentrations of alcohol as type I, and although a few strains grow weakly in wine containing 20 per cent. of alcohol, the majority are unable to grow vigorously when the alcohol content of the medium exceeds 18 per cent. by volume.

Type II comprises 4 strains which ferment xylose in addition to dextrose and levulose, and also form a little acid from sucrose and maltose. This organism also decomposes malic acid, and one strain decomposes citric acid. Two strains have given a weak growth in wine containing 20 per cent. of alcohol.

Type III is represented by two strains. These are the only strains out of all those studied which ferment lactose vigorously. They were isolated from lees and do not grow in wines which contain more than 18 per cent. of alcohol.

Type IV is represented by 15 strains which ferment a number of sugars, including arabinose and xylose, but not lactose or mannose. Ten strains attack malic acid but not citric acid, four strains attack both of these acids, and one strain does not attack either malic or citric acids. One strain caused sliminess when cultured in liquid media.

Type V appears to be the most common, and 62 of the 110 strains are of this type. These strains differ from type IV in their failure to ferment arabinose. Forty-eight strains attack malic but not citric acid, 6 strains attack both acids, 5 strains do not attack either acid, while one strain attacks citric acid strongly and malic acid only slightly.

On the basis of the characters observed, the five types may be described as follows:

Type I.

Morphology: rods 0·4 to 0·6 by 2 to 4 microns, occurring singly, in pairs and in chains and sometimes in very long thread-like chains and filaments. Non-motile. Gram-positive in young cultures but in old cultures some are gram-negative. Without endospores.

Gelatin stab: growth usually poor, but when it occurs is uniform from top to bottom. No liquefaction.

Agar colonies: develop slowly; are small, subsurface, creamy white, irregular in shape.

Agar slant: usually no growth but some strains give a weak filiform growth.

Potato slant: no growth.

Growth in autolysed yeast medium: medium becomes cloudy after 3 to 5 days' incubation, and exhibits a very pronounced silky wavy appearance when shaken gently. After 2 to 3 weeks, the organism settles and forms a compact sediment.

No growth in grape juice, dextrose peptone broth, or bacto-yeast extract medium.

Litmus milk: no growth.

Substances fermented: acid formed in dextose and levulose. Sometimes a little acid formed in sucrose and maltose. Arabinose, xylose, galactose, mannose, lactose, raffinose, glycerol, mannitol, malic acid, citric acid and tartaric acid are not attacked.

Products of fermentation: lactic and acetic acids, carbon dioxide and alcohol are the chief products from dextrose, and lactic and acetic acids, carbon dioxide and mannitol are formed from levulose.

Micro-aerophilic.

Optimum temperature range: in alcohol-free medium, 25 to 30° C. In wine or other media of high alcohol content, optimum temperature range is 20 to 25° C.

Optimum pH range: 4·5 to 5·5. No growth occurs when initial pH of the medium is above 5·8 or below 3·5.

Alcohol tolerance: grows vigorously in wine containing 20 per cent. alcohol. A few strains grow in 21 per cent. alcohol.

Type II.

Morphology: rods 0·6 to 0·8 by 2 to 4 microns, occurring singly, in pairs and often in chains and filaments up to 15 microns in length. Gram-positive. Non-motile. Without endospores.

Gelatin stab: growth uniform from top to bottom. No liquefaction.

Agar colonies: small subsurface, creamy white, irregular in shape.

Agar slant: weak growth.

Potato slant: no growth.

Growth in autolysed yeast medium: turbid after 1 to 2 days. Clears slowly, forming compact sediment.

Litmus milk: no growth.

Substances fermented: acid formed in dextrose, levulose and xylose, and a little acid in galactose, sucrose, and maltose. Malic acid decomposed and some strains decomposed citric acid. Arabinose, mannose, lactose, raffinose, glycerol, mannitol and tartaric acid are not attacked.

Products of fermentation: lactic and acetic acids, carbon dioxide and alcohol from dextrose; lactic and acetic acids, carbon dioxide and mannitol from levulose.

Micro-aerophilic.

Optimum temperature range: in alcohol-free medium, 30 to 35° C. In wine, 20 to 25° C.

Optimum pH range: 4·5 to 5·5. No growth at 3·5, but some strains are able to grow at pH 6·5.

Alcohol tolerance: usually between 18 and 20 per cent., but some strains grow weakly in 20 per cent. alcohol.

Type III.

Morphology: rods 0·6 to 0·8 by 2 to 4 microns, occurring singly and in pairs, and often in chains and filaments up to 15 microns in length. Gram-positive. Non-motile. Without endospores.
Gelatin stab: growth uniform from top to bottom. No liquefaction.
Agar colonies: small subsurface, creamy white, irregular in shape.
Agar slant: weak filiform growth.
Potato slant: no growth.
Growth in autolysed yeast medium: turbid after 1 to 2 days, clears after about 7 days, forming a compact sediment.
Litmus milk: no growth.
Substances fermented: acid in arabinose, xylose, dextrose, levulose, galactose, maltose, sucrose, lactose, and raffinose. Decomposes malic acid. Mannose, glycerol, mannitol, citric acid, and tartaric acid are not attacked.
Products of fermentation: lactic and acetic acids, carbon dioxide and alcohol from dextrose. Lactic and acetic acids, carbon dioxide and mannitol from levulose.
Micro-aerophilic.
Optimum temperature range: in alcohol-free medium, 30 to 35° C. In wine, 20 to 25° C.
Optimum pH range: 5·0 to 5·5. Some growth occurs at all pH values from 3·5 to 7·0.
Alcohol tolerance: between 16 and 18 per cent.

Type IV.

Morphology: rods 0·6 to 0·8 by 2 to 4 microns, occurring singly and in pairs, and often in chains and filaments up to 15 microns in length. Gram-positive. Non-motile. Without endospores.
Gelatin stab: growth uniform from top to bottom. No liquefaction.
Agar colonies: small subsurface, creamy white, irregular in shape. Also a few small, round, flat surface colonies.
Agar slant: weak filiform growth.
Potato slant: no growth.
Growth in autolysed yeast medium: turbid after 1 to 2 days, clears after 7 to 10 days. Most strains form a compact sediment, but some form a loose granular sediment.
Litmus milk: no growth.
Substances fermented: acid in arabinose, xylose, dextrose, levulose, galactose, maltose, sucrose and raffinose. Most strains decompose malic acid, some also decompose citric acid, while some do not attack either malic or citric acids. Lactose, mannose, glycerol, mannitol and tartaric acid are not attacked.
Products of fermentation: lactic and acetic acids, carbon dioxide and alcohol from dextrose. Lactic and acetic acids, carbon dioxide and mannitol from levulose.
Micro-aerophilic.

Optimum temperature range: in alcohol-free medium, 30 to 35° C. In wine, 20 to 25° C.

Optimum pH range: 4·5 to 6·0. Some growth occurs at all pH values from 3·5 to 7·0.

Alcohol tolerance: usually between 18 and 20 per cent., but some strains grow weakly in 20 per cent. alcohol.

Type V.

Morphology: rods 0·6 to 0·8 by 2 to 4 microns, occurring singly and in pairs, and often in chains and filaments up to 15 microns in length. Gram-positive. Non-motile. Without endospores.

Gelatin stab: growth uniform from top to bottom. No liquefaction.

Agar colonies: small subsurface, creamy white, irregular in shape. Also a few small round, flat surface colonies.

Agar slant: weak filiform growth.

Potato slant: no growth.

Growth in autolysed yeast medium: turbid after 1 to 2 days, clears after 7 to 10 days. Most strains form a compact sediment, but some form a loose granular sediment.

Litmus milk: no growth.

Substances fermented: acid in xylose, dextrose, levulose, galactose, maltose, sucrose, and raffinose. Most strains decompose malic acid, some also decompose citric acid, while a few do not attack either citric or malic acid. Arabinose, mannose, lactose, glycerol, mannitol, and tartaric acid are not attacked.

Products of fermentation: lactic and acetic acids, carbon dioxide and alcohol from dextrose. Lactic and acetic acids, carbon dioxide and mannitol from levulose.

Micro-aerophilic.

Optimum temperature range: in alcohol-free medium, 30 to 35° C. In wine, 20 to 25° C.

Optimum pH range: 4·5 to 6·0. Some growth occurs at all pH values from 3·5 to 7·0.

Alcohol tolerance: usually between 18 and 20 per cent., but some strains grow weakly in 20 per cent. alcohol.

(4) Systematic Relationships of the Bacteria

From a consideration of the descriptions of the bacteria, it is evident that all five types belong to the gas-producing or heterofermentative section of the genus *Lactobacillus*. This section of the genus has recently been reclassified by Pederson (1938), who found that many of the types which were previously listed as separate species are so much alike that they

should be considered as merely different varieties of a single species. Eighteen species, described by different workers and listed in the fourth edition of Bergey's Manual, have been grouped by Pederson into four species and a fifth group which includes several bacteria not yet described in sufficient detail to enable their relationships with other bacteria to be determined. The chief characters used by Pederson in defining species are the optimum temperature range for growth and the ability to ferment different sugars, particularly arabinose, mannose, lactose, sucrose, and raffinose.

Since Pederson's classification of the lactobacilli has received wide recognition it is of interest to compare the descriptions given in the preceding section with those of the species recognized by Pederson. It has been found that only some of the five types isolated from fortified wines bear a close resemblance to any of the species of Pederson's classification.

Type I differs from the other four types in morphology, fermentation reactions, temperature relationship, and alcohol tolerance, and is also more exacting as regards growth requirements, including pH and composition of the medium. This organism does not correspond with any of the species defined by Pederson nor with descriptions published by other workers. Some degree of similarity may be noticed between the description of Type I and the description of *Bacterium gracile* published by Müller-Thurgau and Osterwalder (1913). The two organisms resemble one another in morphology, in their temperature relationships, and in their comparative inactivity towards most sugars other than dextrose and levulose. However, *Bacterium gracile* is described as attacking malic and citric acids very vigorously and as being able to tolerate only 10 per cent. by volume of alcohol, whereas Type I grows quite well, though slowly, in 20 per cent. of alcohol, and none of the strains which have been studied attack malic or citric acids.

Types III and IV bear a closer resemblance to *Lactobacillus buchneri* (Henneberg emend Pederson) than to any of Pederson's other species, but they differ from *L. buchneri* in having a slightly lower optimum temperature, in their failure to ferment mannose and, in the case of Type IV, failure to ferment

lactose. *Bacterium mannitopoeum* (Müller-Thurgau) is included in *L. buchneri* by Pederson, but, according to the description published by Müller-Thurgau and Osterwalder, *B. mannitopoeum* ferments lactose only very weakly, if at all, and has an optimum temperature range of 26·4 to 34° C. Types III and IV are able to tolerate greater concentrations of alcohol than any of the strains of *B. mannitopoeum* which Müller-Thurgau and Osterwalder described, but in other respects Type IV resembles the original description of this organism very closely, while Type III differs from it only in its vigorous fermentation of lactose. It is suggested that both of these types belong to this group and that they may be regarded as varieties of *Lactobacillus buchneri.*

The relationship of Types II and V to previously described species is rather uncertain. These organisms resemble *Bacterium intermedium* (Müller-Thurgau and Osterwalder) in their failure to ferment arabinose and their ability to ferment xylose, but they differ from that organism in failing to ferment lactose, while Type II also differs from it in being less active towards several other sugars, notably galactose and raffinose. The bacteria which fail to attack arabinose are grouped by Pederson under the specific name *Lactobacillus fermentum* (Beijerinck), and both *B. gayonii* (Müller-Thurgau and Osterwalder) and *B. intermedium* (Müller-Thurgau and Osterwalder) are included as varieties of this species. According to Pederson, however, one of the chief characteristics of this species is its high optimum temperature range, namely 35° to 40° C. or higher. In view of the lower optimum temperature range of Types II and V and their failure to ferment lactose, their inclusion in the species *L. fermentum*, as it is defined by Pederson, is hardly warranted.

It is perhaps debatable whether Types IV and V should be further subdivided according to the ability of different strains to attack malic and citric acids, for Müller-Thurgau and Osterwalder relied chiefly on this character to distinguish *B. gayonii* from *B. intermedium.* In the present case, however, such a subdivision does not seem to be warranted, for no correlation between the ability to attack these acids and other important characters has been observed. Moreover, the extent to which

different strains attack these acids varies considerably and the strains do not fall into well-defined groups in this respect.

It is not altogether surprising that some of the organisms here described do not readily fit into Pederson's scheme of classification, for no bacteria which had been isolated from fortified wines were considered when this scheme was drawn up. Naturally, newly isolated strains of bacteria do not always exhibit characteristics in accordance with existing schemes of classification which are necessarily drawn up after a study of a limited number of strains. However, the practice of describing as a new species every strain which differs slightly from previously described species, merely creates confusion and renders any scheme of classification unworkable. The published descriptions of different species should rather be considered as indicating typical strains only, and further strains isolated may be expected to exhibit minor variations from these. It therefore seems desirable that only those strains which differ very greatly from described species should be considered as new species.

Of the five types of bacteria described in the present study only Type I appears to differ sufficiently from the species listed in Pederson's scheme to be considered as a new species. Types III and IV appear to be varieties of *L. buchneri*, but while Types II and V resemble *L. fermentum*, in some respects they do not correspond with Pederson's definition of that species.

3. Effects of the Bacteria on Wines as Determined by Inoculating with Pure Cultures.

In a previous section comparisons were made between spoiled and sound wines. These comparisons were based on data obtained from the examination of samples of sound wines and wines which had undergone bacterial spoilage under industrial conditions. Such comparisons indicate the characteristics by which the spoiled wines may be recognized, but they do not give accurate information concerning the effects of the bacteria on the composition of the wine, for the original composition of the spoiled wine was, of course, not known. For the purpose of making such comparisons it is assumed that the

composition of the spoiled wines originally resembled that of sound wines of similar type, but this assumption is valid only within certain limits. It is shown in a later section that wines with a low acidity are more liable to bacterial attack than wines with a high acidity. Thus it is unlikely that the original pH and titratable acidity of the spoiled wines were similar to those of the sound wines.

The results discussed in the present section were obtained by inoculating wines with pure cultures of bacteria and analysing both the inoculated wines and uninoculated controls, after a suitable period of incubation. The data thus obtained show the effects of the bacteria on the composition of the wine under a particular set of conditions. Thus it can be shown that a particular bacterium is actually the cause of the type of spoilage under discussion. Furthermore, comparisons can be made between the changes brought about by different bacteria when grown in different portions of the same wine or between the changes brought about in different types of wines by a single strain of bacterium.

(1) Spoilage Due to Growth of the Bacteria in Fortified Wines

Several wines were inoculated with pure cultures of bacteria in order to determine the changes which occur in the composition of wines during bacterial spoilage. The wines which were chosen for this purpose had been shown by preliminary trials to support bacterial growth. In some cases the acidity and the alcohol content of these wines were reduced somewhat in order to ensure vigorous growth of the bacteria. The wines were sterilized by means of a Chamberland porcelain filter and were poured into sterile bottles. They were then inoculated with a few drops of a vigorous culture in susceptible wine, and the bottles were closed with waxed corks. The inoculated wines, and also uninoculated controls, were incubated at 23° C. for twelve weeks and were then analysed.

The results presented in Table 2 indicate the changes brought about by *Lactobacillus* type I when grown for twelve weeks in a sweet white wine.

TABLE 2

Effect of Lactobacillus Type I on a Sweet White Wine; 12 weeks growth at 23° C.

	Controls.	Inoculated.
Alcohol, per cent. by volume	19·0	17·8
Total titratable acidity as g/l tartaric acid	4·25	7·54
Volatile acidity as g/l acetic acid	0·36	2·15
Fixed acidity (by difference) g/l tartaric acid	3·80	4·85
Reducing sugar as g/100 c.c. dextrose	11·0	9·4
pH	4·30	3·90
Condition	bright	very turbid
Microscopical examination	no bacteria	bacteria very numerous

From these results it can be seen that the bacterium attacked the sugars and produced both volatile and non-volatile acids. This increase in acidity is reflected in the lowering of the pH of the inoculated wines. There is also an apparent reduction in the alcohol content of the wine during bacterial growth. A loss of alcohol if often noticed in such experiments and is probably due to evaporation, for the evolution of CO_2 makes it difficult to keep the bottles tightly corked during a long period of incubation. The wine referred to in Table 2 was incubated in four-ounce bottles and the loss of alcohol was greater from these than from the thirteen-ounce bottles used in several other experiments.

Table 3 shows the changes brought about by three different bacteria (Types I, IV, and V) when grown in different portions of the same wine. In this case a sweet white wine was used, and the alcohol content was lowered to 16 per cent. by volume and the pH raised to 4·4 by the addition of water and potassium carbonate respectively.

It can be seen from this table that the bacteria all developed very vigorously and effected considerable changes in the composition of the wine. These changes are very much greater than those which are usually associated with bacterial spoilage of wines under industrial conditions, but they are essentially the same type of changes. The very intense development of the bacteria in this experiment may be attributed in part to the vigour of the cultures used for inoculation, but is due chiefly

to the composition of the wine used. The close similarity between the changes brought about by the different bacteria is at once evident from the table. Each of the three bacteria attacked the sugars present in the wine and formed lactic and acetic acids, mannitol, and also carbon dioxide gas. The increase in both fixed and volatile acidity is also reflected in a very marked fall in the pH of the wine. Thus, although three different bacteria were involved, the type of spoilage produced in a sweet wine was the same in each case. In this experiment the loss of alcohol from the cultures was much less than in the previous experiment (cf. Table 2) although the bacterial development and the increase in acidity were much greater. This is probably due to the fact that the second experiment was carried out in 13-ounce bottles instead of the 4-ounce bottles used previously, and losses by evaporation were lower from the larger volume of liquid.

TABLE 3

Changes brought about in a Sweet White Wine by Different Types of Bacteria.

		Inoculated with		
	Control.	Type I.	Type IV.	Type V.
Alcohol per cent. by vol.	16	15·7	15·7	15·5
Total acidity as g/l tartaric acid	2·29	15·41	15·05	14·9
Volatile acidity as g/l acetic acid	0·17	5·71	5·89	5·6
Fixed acidity (by difference) as g/l tartaric acid	2·08	8·27	7·70	7·86
Lactic acid g/l	trace	7·03	6·57	6·61
Reducing sugar as dextrose g/100 c.c.	6·86	2·01	2·43	2·54
pH	4·40	3·46	3·47	3·50
Nature of the volatile acid	acetic	acetic	acetic	acetic
Presence of mannitol	—	+	+	+
Condition	bright	very turbid, with deposit on bottom of bottles.		
Microscopical examination	no bacteria	bacteria very numerous.		

Results of the analysis of wine in which bacteria belonging to Types II and III had been grown are shown in Table 4. The alcohol content of the wine used in this experiment was reduced to 14 per cent. to ensure vigorous growth. It appears that these bacteria, when grown in sweet wines, both bring about the same type of spoilage as do the other types. How-

ever, the two strains of Type III which were isolated failed to grow in wines containing 18 per cent. of alcohol, and it seems unlikely that these are able to cause spoilage of fortified wines.

TABLE 4

Effect of Lactobacillus *Type II and Type III on a Sweet White Wine (density 3° Bé). 8 weeks' growth at 23° C.*

		Inoculated with	
	Control.	Type II.	Type III
Alcohol per cent. by vol.	14	13·9	13·7
Total titratable acidity as g/l tartaric acid	3·52	7·65	10·85
Volatile acidity as g/l acetic acid	0·40	2·24	3·73
Fixed acidity as g/l tartaric acid	3·02	4·85	6·19
pH	4·2	3·8	3·65
Condition	Brilliant	Very turbid	Very turbid
Microscopical examination	No bacteria	Bacteria very numerous	

The results presented in Tables 2, 3, and 4 indicate that the five types of bacteria when grown in sweet wine, all bring about the same type of spoilage as occurs in Australian fortified wines under industrial conditions. It has been observed that Type I is more often associated with the spoilage of highly fortified wines than are the other types of bacteria, but this appears to be due entirely to the greater tolerance of Type I towards alcohol, for it has been shown that the other types of bacteria have much the same effect on the wine provided the alcohol content is low enough to enable them to grow.

The spoilage which these bacteria bring about when growing in fortified wines appears to resemble the spoilage of fortified wines occurring in South Africa (Niehaus, 1930, 1932) and California (Cruess, 1934, 1935a), but differs considerably from 'tourne' and other types of spoilage occurring in dry wines in Europe. Of the various types of bacterial spoilage which have been described by European writers, the lactic acid spoilage described by Müller-Thurgau and Osterwalder (1913, 1918) bears the closest resemblance to the type of spoilage under discussion, but the alcohol content of the wines mentioned by Müller-Thurgau and Osterwalder was much lower than that of our fortified wines.

(2) Spoilage Due to Growth of the Bacteria in Dry Wines

In wines of low sugar content the extent of spoilage varies according to the amount of sugar present. When this amounts to about 0·5 per cent. or more, as in many fortified dry white wines, the type of spoilage is essentially similar to that occurring in sweet wines, although the amounts of acetic and lactic acids produced are usually much less than in the sweet wines.

Some bacterial development may also occur in wines containing quite small amounts of sugar, and lactic acid bacteria have been observed in many samples of dry wines and dry wine lees. In the majority of these samples very little evidence of spoilage could be detected, but one dry wine had a pronounced 'mousy' odour and a volatile acidity of 2·04 grms. per litre (as acetic acid). On analysis this wine was found to contain 2·4 grms. of reducing sugar (as dextrose) per litre and so probably contained an appreciable amount of sugar at the time of infection.

In the laboratory strains of bacteria representing Types III, IV, and V have been grown in wines containing only 0·1 per cent. of reducing sugar, but the growth obtained was never vigorous and no changes were detected in the composition of the wines. During the growth of the bacteria the wines became turbid, however, and remained in this condition for periods which varied from two to eight weeks. Eventually the bacteria settled to the bottom of the container and the wine became clear and showed very little evidence of bacterial spoilage.

(3) Spoilage Due to Growth of the Bacteria in Must

All strains of *Lactobacillus* Type I have consistently failed to grow in unfermented grape juice except after the addition of autolysed yeast. Many strains of the other types grow quite readily in grape juice, however, and these may cause serious spoilage before or during the primary fermentation.

In an experiment, several 150 ml. portions of grape juice were placed in 8-ounce bottles and inoculated with a wine yeast. Duplicate bottles were then inoculated with different strains of bacteria and were incubated at 23° C. for ten days.

At the end of this period the fermented liquids were examined for bacterial growth and the volatile acidity of each was determined.

As can be seen from the results in Table 5, bacterial growth was accompanied by a considerable increase in the volatile acidity of the wines, although in some cases the appearance of the wine was not greatly affected.

TABLE 5

Spoilage of Wine due to the Growth of Bacteria during the Primary Fermentation.

Organism.	Volatile acidity. g/l acetic acid.	Appearance of wine.	Microscopical examination of the lees.
Lactobacillus			
Type I	0·60	Clear	No bacterial growth
Type II	0·56	"	" " "
Type III	1·09	"	Bacteria very numerous
Type IV str. L15	1·75	Turbid	" " "
Type IV str. L79	0·84	Clear	Bacteria fairly numerous
Type V	1·18	Slightly turbid	Bacteria very numerous
Control (yeast only)	0·60	Clear	No bacteria

This type of spoilage is usually not considered as 'bacterial spoilage of fortified wines.' It seems obvious, however, that if bacteria grow in the fermenting must, the wines may be spoiled before they are fortified.

CHAPTER III

THE EFFECTS OF VARIOUS FACTORS ON THE GROWTH OF THE BACTERIA IN WINES

It has been observed that some wines are much more liable to bacterial spoilage than are others of similar type. A large number of fortified wines have been inoculated and incubated, but bacterial growth has occurred in only a few of them. It is, of course, well known that wines of similar type differ appreciably in composition, and it appears that such differences as occur in the composition of fortified wines are of very great importance in relation to bacterial development.

The extent to which different wines may be affected by inoculation is illustrated by the following experiment:

Three different wines of the same type (sweet white) and made in the same winery, were inoculated with *Lactobacillus* Type I. The wines were examined after remaining at room temperature (19°–23° C.) for ten weeks, with the following results:

Wine A Unchanged; no bacterial growth.

Wine B Very turbid: titratable acidity increased by 3·2 grm. per litre as tartaric acid.

Wine C Slightly turbid; titratable acidity increased by 0·3 grm. per litre as tartaric acid.

Differences in the susceptibility of wines to bacterial attack are obviously of very great practical importance. For this reason, the effects of different factors on the growth of the bacteria in wine and in artificial media have been investigated. In this section of the investigations, particular attention has been devoted to *Lactobacillus* Type I, for this organism, because of its high alcohol-tolerance, is of great importance in the spoilage of fortified wines. In many respects the growth requirements of the five types of bacteria are similar, but Type I is rather more exacting than the other types. A wine which supports the growth of Type I will also support the growth of

the other types if alcohol is not a limiting factor, but some wines which contain less than 18 per cent. of alcohol support the growth of Types II, III, IV, and V but not that of Type I.

The increased titratable acidity of cultures, as compared with uninoculated controls, has been used as a measure of bacterial activity, throughout this section of the investigations.

1. Acidity of the Wines

The importance of acidity as a factor influencing bacterial spoilage of wine is realized in a general way by most wine-makers. However, Ventre (1925) appears to have been the first to draw attention to the fact that the influence of acidity on bacterial spoilage is more closely related to the pH than to the titratable acidity of the wine.

The pH relationships of the different types of bacteria have already been mentioned. The five types all grow most vigorously between pH 4·5 and pH 5·5, but they differ somewhat in their ability to grow at pH values above and below this range. Type I has failed to grow in wine or other media when the pH has been adjusted to above 5·8 or below 3·5, but most strains of the other types are able to grow at all pH values between 3·5 and 7·0.

The pH values of 276 fortified wines have been determined. The highest and lowest pH values recorded in this series were 4·35 and 3·05 respectively. The pH values of 197 wines (71·5 per cent. of those examined) were between 3·5 and 4·0, while 18 wines (6·5 per cent.) had pH values below 3·5, and 61 wines (22 per cent.) had pH values above 4·0. Thus the range which includes the pH values of normal fortified wines also includes the lower portion of the pH range over which the different types of bacteria are able to grow. Consequently, variations which occur in the pH of fortified wines may be expected to have an important influence on bacterial growth.

The present discussion is concerned only with the direct effect of the pH of the wine on bacterial growth. The indirect effect of the pH of the must on the composition of the wine, and on bacterial growth, will be discussed in a later section.

The results presented in Table 6 show the effect of pH on the growth of three strains of *Lactobacillus* Type I in a sweet wine which contained 20 per cent. of alcohol by volume. The original pH of the wine was 4·05, but it was adjusted with citric acid and with normal sodium hydroxide solution for the purpose of the experiment. The wine was sterilized by filtration, placed in 4-ounce bottles and inoculated, and the bottles were closed with waxed corks. After being incubated at 23° C. for 12 weeks, the contents of the bottles were examined for bacterial growth and for increased titratable acidity. As may be seen from the table, the growth of each strain was very much more vigorous at the higher pH values. At pH 3·6 only very weak growth occurred, and the wine was only slightly affected, while no detectable growth occurred at pH 3·4.

TABLE 6

Effect of pH on the Growth of Three Strains of Lactobacillus *Type I in a Fortified White Wine.*

pH of wine when inoculated.	Increase in titratable acidity as g/l tartaric acid. Strain Lla.	Strain Llb.	Strain Llc.
3·4	—	O	—
3·6	0·2	T	0·2
3·8	1·5	0·7	1·0
4·0	1·9	2·4	2·6
4·2	2·7	3·4	3·4
4·4	3·2	3·7	4·1

O = not determined; — = no growth; T = slight growth but no appreciable production of acid.

In some wines the bacteria have produced sufficient acid to lower the pH to 3·5, but all attempts to culture them in wine with the pH adjusted to 3·5 have been unsuccessful. Some strains of Types III, IV, and V have been grown in autolysed yeast medium at pH 3·4, but the lower limit of the pH range over which fortified wines may be effectually inoculated with any of the five types appears to be very close to pH 3·6. It is of interest to compare this figure (pH 3·6) with the value of pH 3·4, which Ventre (1925, 1931) gives as the lower limit for the growth of the bacteria causing 'tourne' in wines. According to Brémond (1937) the bacteria

responsible for 'tourne' are unable to develop in Algerian wines which have a pH lower than 3·5. In wines which have a lower pH the bacteria, if present, remain in the lees and do not multiply to any appreciable extent. Brémond claimed that determination of the pH of wines is a simple and certain method of ascertaining whether the wines will remain sound during storage.

2. Temperature

It has already been pointed out that the optimum temperature range for the growth of Type I in autolysed yeast medium lies between 25° and 30° C., while Types II, III, IV, and V are favoured by higher temperatures and grow most vigorously between 30° and 35° C. On several occasions, however, samples of wine in which bacterial growth was just commencing have been received at the laboratory and have been incubated. It has been observed that when such samples are incubated at a temperature of 30° C. bacterial growth is very weak or ceases entirely, whereas bacterial growth continues vigorously in similar samples left at room temperature (18° to 23° C.). The results of experiments with wine and artificial media which contained alcohol, indicate that the optimum temperature range for the growth of the five types of bacteria in the presence of 15 to 20 per cent. of alcohol lies between 20° and 25° C.

The effects of different temperatures on acid production by Type V in media containing different amounts of alcohol are shown in Table 7. Portions of autolysed yeast medium, to which different amounts of alcohol had been added, were placed in small bottles and inoculated. The bottles were sealed with waxed corks, and duplicate cultures in each medium were incubated at each of the temperatures indicated in the table. In the alcohol-free medium, some growth occurred at all temperatures between 14° and 36°, and the optimum temperature, as evidenced by the appearance of turbidity and by acid production in seven days, was in the vicinity of 32° C. In the presence of alcohol, growth was much slower at all temperatures. Growth in 10 per cent. alcohol was quite appreciable after seven days, but in order to obtain satisfactory

growth in 15 per cent. and 20 per cent. alcohol, the periods of incubation were extended to 28 days and 56 days respectively. For this reason, the figures representing acid production in Table 7 were obtained after periods of incubation which differed according to the alcohol content of the media.

TABLE 7

Effect of Temperature on Growth of Lactobacillus *Type V in Different Concentrations of Alcohol.*

	Increase in titratable acidity as g/l tartaric acid.			
Temp. °C.	Alcohol-free 7 days' growth.	10 per cent. alcohol 7 days' growth.	15 per cent. alcohol 28 days' growth.	20 per cent. alcohol 56 days' growth.
7	—	—	—	—
11	—	—	—	—
14	T	—	—	—
15·5	0·43	T	—	—
18	1·07	0·10	T	T
20	1·30	0·23	0·10	T
22	2·45	0·38	1·00	0·90
23·5	2·90	0·53	1·60	1·55
25	3·21	1·15	0·25	T
27	3·89	1·60	T	—
29	4·54	0·99	—	—
32	5·01	T	—	—
36	4·35	—	—	—

— signifies no apparent growth within the period indicated.
T signifies slight turbidity produced but no appreciable change in titratable acidity.

As may be seen from the table, the maximum temperature for growth, and also the temperature at which acid production was greatest within the periods specified, varied according to the alcohol content of the medium. The figures presented in Table 7 represent acid-production during the early stages of growth rather than the maximum amounts of acid formed, for the cultures were still growing actively when they were titrated. The optimum temperature range determined in this way agreed very closely with the range over which initial growth was most vigorous as determined by visual inspection of the cultures.

The temperature at which acid production is greatest also varies according to the length of the incubation period. The results presented in Table 8 represent the amounts of acid produced at different temperatures, during different periods. Cultures of *Lactobacillus* Type V were grown in autolysed yeast medium which contained 10 per cent. of alcohol. Duplicate cultures were incubated at each of the temperatures listed in the table, and portions of each culture were titrated after periods of 7, 14, and 28 days.

TABLE 8

Acid Production by Lactobacillus *Type V during Different Periods at Different Temperatures.*
Autolysed Yeast Medium Containing 10 per cent. Alcohol.

Temperature. °C.	Increase in titratable acidity as g/l tartaric acid. After 7 days.	After 14 days.	After 28 days.
7	—	—	—
11	—	—	T
14	—	T	1·50
15·5	T	1·05	3·30
18	0·10	1·50	3·45
20	0·23	2·10	3·85
22	0·38	2·25	4·80
23·5	0·53	2·40	5·20
25	1·15	2·55	5·10
27	1·60	2·40	4·20
29	0·99	2·25	4·10
32	T	1·95	3·75
36	—	—	—

— = no apparent growth within the period indicated.
T = slight turbidity produced but no appreciable change in titratable acidity.

After 28 days, the cultures which had been incubated at 25° C. and higher temperatures had become clear, and acid production had practically ceased in these. Cultures which had been incubated at the lower temperatures, however, still exhibited some degree of turbidity, although the majority of the bacteria had settled out of the liquid. At low temperatures, growth and acid production proceed slowly over a long period. For this reason, the temperature at which the maximum amount

of acid is produced cannot be defined easily, but it is evident that this temperature is lower than that at which the greatest amount of acid is produced during the early stages of growth.

Müller-Thurgau and Osterwalder (1913), using pear juice as a culture medium, found that the optimum temperature for acid production in 10 days, by *B. mannitopoeum*, was between 26° and 34° C. After a period of 79 days, however, the greatest amounts of acid were produced in those cultures which had been incubated at temperatures between 18° and 23° C. Similarly, Dorn and Rahn (1939) have shown that the figures obtained for the optimum temperature range of the lactic acid streptococci differ according to the way in which the optimum temperature is defined and determined. Production of acid by suspensions of *Str. lactis* was most rapid at 40° C., but the greatest amount of acid was formed at 30° C.

The effect of the period of incubation on the optimum temperature for acid-production can be explained by assuming that the optimum temperature depends on the pH of the medium. Dorn and Rahn (loc. cit.) point out that the temperature at which the greatest production of acid occurs is, in reality, the temperature at which the acid-tolerance of the organism is greatest. It appears that the adverse effects of high acidity, like those of high alcohol concentration, increase with rise in temperature, even within the range 20° to 30° C. From observations made, it seems likely that the optimum temperature range, as measured by maximum production of acid over a long period, is not greatly affected by the alcohol content of the medium, and that this range is in the vicinity of 20° to 25° C. for the five types of bacteria under discussion.

It is commonly supposed that bacterial spoilage of fortified wines is favoured by high temperatures. The special significance of high temperatures during the primary fermentation and early storage will be discussed in a later section, but the results of a large number of tests indicate that high temperatures (i.e above 25° C.) are not favourable for the growth of the bacteria in fortified wines. None of the strains studied has proved capable of growing in fortified wines which were incubated at 30° C., and growth and acid production in such wines have always been less vigorous at 27° than at tempera-

tures between 20° and 25° C. On the other hand, growth at 15° C., although very slow, is often quite appreciable, and wines stored at such a temperature may undergo considerable spoilage over a long period.

3. Accessory Growth Substances

Bacteria belonging to the genus *Lactobacillus* are notoriously exacting in their growth requirements. For normal growth these bacteria require not only a supply of ordinary nutrients in available form, but also small amounts of accessory growth substances. Some of these accessory growth substances are identical with some of the vitamins required by man and animals, while others are, so far as is known, required only by certain bacteria. Some are of unknown composition, while others have been identified and their chemical composition has been determined. However, the various substances all have this feature in common—they are required in only minute amounts.

The requirements of the lactobacilli for accessory growth substances are not fully understood as yet, but as a result of recent investigations by a number of different workers, much valuable information has been made available. Pantothenic acid, an acidic substance of wide biological occurrence, was found to be essential for the growth of 13 species by Snell, Strong, and Peterson (1937, 1938, 1939). This substance is required by all species of *Lactobacillus* tested, and is probably required by the whole genus. On the other hand some substances are required by some species and not by others. In this category are adermin or vitamin B_6 (Moller, 1939; Snell and Peterson, 1940), nicotinic acid (Snell, Strong, and Peterson, 1939), biotin (Moller, 1939), and riboflavin or vitamin B_2 (Orla-Jensen *et al.*, 1936; Snell and Strong, 1939). Other substances as yet not identified have been found necessary for some species by Orla-Jensen and co-workers (1936) and by Otani (1936).

It is worthy of note that closely related species may differ in their requirements for accessory growth substances. Thus Snell and Strong (1939) found that riboflavin was required by *L. gayonii* but not by *L. mannitopoeus*.

Most of the substances which have been reported as stimulating the growth of lactic acid bacteria are present in appreciable amounts in autolysed yeast, and many of the lactic acid bacteria show a marked increase in growth when quite small amounts of autolysed yeast are added to basal media. The species which cause spoilage of fortified wines also show a marked response to the addition of autolysed yeast to the culture medium. Several wines which had previously shown no response to inoculation were successfully infected after the addition of 0·5 to 1·0 per cent. of autolysed yeast. A similar enrichment greatly improves peptone solution and grape juice as culture media for these bacteria.

The actual substances which are responsible for the stimulating effect of autolysed yeast have not been identified in the present investigations. However, it has been found that the activity of autolysed yeast was only slightly reduced by heating for two hours at 120° C. in the autoclave at pH 4, whereas activity was completely destroyed by a similar heating at pH 8·5. Treatment of the autolysed yeast with 80 per cent. alcohol or with active carbon (norite) also caused a marked decrease in activity, particularly in respect to *Lactobacillus* Type I. On the other hand, treatment with one per cent. tannic acid had very little effect on the activity of the autolysate.

It is interesting to note that all strains of Type I have failed to grow in grape juice unless autolysed yeast had been added, whereas many strains of Types III, IV, and V grew quite readily in grape juice and also in some wines which did not support the growth of Type I. It seems likely that Type I requires one or more factors which are not essential for the growth of all strains of the other types. One accessory growth substance which is abundant in autolysed yeast, but of which grape juice was found to contain very little by Flavier (1939), is riboflavin. In view of the findings of Snell and Strong (1939) concerning the requirements of different species for this substance, it is possible that Type I is more dependent on the presence of riboflavin in the medium than are the other types. However, direct evidence on this point is not at present available.

Occurrence of Accessory Growth Substances in Wines

It appears that some of the factors required by the bacteria are not present in adequate amounts in all fortified wines. It is evident, however, that the essential growth substances which are lacking in some fortified wines, in grape juice, and in peptone solution, are present in autolysed yeast. This point is of great importance in relation to the spoilage of fortified wines, for the yeast cells in the gross lees and in the young wine are a potential source of the growth substances, and these may be liberated in the wine if autolysis occurs.

Many oenologists believe that prolonged contact with the gross lees renders a wine more subject to bacterial attack. Niehaus (1932) found that prolonged contact with the gross lees increased the susceptibility of South African wines to bacterial spoilage, and he attributed this to the liberation of certain substances during autolysis of the yeast cells. It is a matter of common observation, however, that not all those wines which are racked late, and therefore are in contact with the gross lees for a long time, become infected with bacteria. In the laboratory it has been found that many of the late-racked wines cannot be infected, even after adjustment of the pH, unless small amounts of autolysed yeast are added. It seems evident therefore, that the length of time a wine is in contact with the gross lees is not the only factor concerned, but that the yeast cells undergo autolysis much more readily in some cases than in others.

Autolysis, being an enzymatic process, is influenced by a number of factors. Two of the most important of these factors are pH and temperature, and both of these may vary appreciably in the contents of different fermentation and storage tanks. According to Waksman and Davison (1926), autolysis of yeast proceeds most rapidly at a reaction of approximately pH 6, and the optimum temperature for this process is usually given as 45° to 50° C. Although these conditions would not normally occur in the fermentation or storage tanks in a winery, it was thought that such variations as occur might have important effects on the autolysis of the yeast cells in the lees. The effect of the length of time the wine is in contact with the yeast cells, and also the effects of the pH and temperature of

the wine during this period, on the susceptibility of the wine to bacterial spoilage, have been investigated in the following experiments.

a. *The Effect of pH and Time of Racking.* Eighty litres of fresh grape juice (Grenache) with a pH of 3·8 and a density of 13·5° Bé, were divided between forty large glass bottles, the necks of which were plugged with cotton-wool. Potassium metabisulphite was added to the contents of each bottle at the rate of 0·2 grm. per litre. The forty bottles were then arranged in three groups:

A comprising 8 bottles
B comprising 24 bottles
C comprising 8 bottles

The pH of group A was raised to 4·3 by the addition of potassium carbonate, the pH of group C was reduced to 3·3 by the addition of tartaric acid, while the pH of group B was allowed to remain at 3·8. One per cent. of an active yeast culture in grape juice was added to each bottle, and fermentation was allowed to proceed at room temperature (20–30° C.). Fermentation was arrested by fortification when the density had been lowered to approximately 5·5° Baumé. The contents of each bottle were fortified to a strength of 20 per cent. alcohol by volume with rectified grape spirit. After fortification, group B was subdivided into three further groups and the pH of these was adjusted as follows:

B_1—pH raised to 4·3.
B_2—pH allowed to remain at 3·8.
B_3—pH lowered to 3·3.

Thus there resulted five groups each comprising eight bottles:

A —Fermented and left on the lees at pH 4·3.
B_1—Fermented at pH 3·8 and left on the lees at pH 4·3.
B_2—Fermented and left on the lees at pH 3·8.
B_3—Fermented at pH 3·8 and left on the lees at pH 3·3.
C —Fermented and left on the lees at pH 3·3.

Two days after fortification the cotton-wool plugs were removed and the bottles were closed by means of waxed corks in order to check loss of alcohol by evaporation. Within each of the five groups the contents of duplicate bottles were racked

and filtered (Whatman No. 2 paper) at intervals of 2 weeks, 4 weeks, 8 weeks, and 12 weeks after fortification. Total nitrogen determinations were carried out on the contents of each of the forty bottles by the micro-Kjeldahl method, and samples of each of the wines were also tested for susceptibility to bacterial spoilage. The samples were placed in sterile 4-ounce bottles and inoculated with one drop of a vigorous culture of *Lactobacillus* Type I after the pH values of all the wines had been adjusted to 4·2 in order to obviate the direct effect on bacterial growth of differences in the pH values of the wines. The bottles were corked tightly and incubated at 23° C. for a period of 12 weeks, by which time considerable differences between the amounts of growth in different samples were noticed. The increased titratable acidity of the inoculated samples, as compared with uninoculated controls, was determined, and the results are summarized in the last column of Table 9.

As can be seen from Table 9 the experimental wines all contained much less total nitrogen than do most Australian sweet wines made under industrial conditions. Although the effects of the different treatments on the nitrogen content of the wines were not great, the total nitrogen content of the wines was increased slightly by delayed racking and also by contact with the lees at pH 4·3.

The results presented in Table 9 indicate that the pH of the must has an important effect on the susceptibility of the resulting wine to bacterial spoilage. This effect is distinct from the effect of the pH value of *the wine* on bacterial growth, for at the time of inoculation the pH of all samples was the same. Comparisons between the amounts of acid produced in the different groups of wines indicate that high pH values, both during the primary fermentation and during storage up till the first racking, favoured the growth of the bacterium in the resulting wine. The wines of group A supported a more vigorous growth of the bacterium than did the wines of group B_1, although the wines of these two groups differed in pH only during the primary fermentation, a period which lasted seven days. On the other hand, the wines of group B_1 supported a more vigorous growth of the bacterium than did the

wines of group B_2, although the wines of these two groups were fermented at the same pH and differed in pH only after fortification.

TABLE 9

The Effects of pH of the Must and Time of Racking on the Susceptibility of Wines to Bacterial Spoilage.

All figures are means of duplicate treatments.

	pH treatment.	Time on gross lees (weeks)	Total N (mgm./l)	Increase in titratable acidity after inoculating at pH 4·3 and incubating at 23° C. for 12 weeks (as grm./l tartaric acid).
Group A	Fermented and left on the lees at pH 4·3	2	117	0·83
		4	118	1·43
		8	123	1·08
		12	127	1·52
	Mean for Group A		*121*	*1·23*
Group B_1	Fermented at pH 3·8 and left on the lees at pH 4·3	2	104	0·80
		4	109	0·91
		8	110	1·00
		12	110	0·83
	Mean for Group B_1		*108*	*0·89*
Group B_2	Fermented and left on the lees at pH 3·8	2	104	0·41
		4	103	0·70
		8	102	0·53
		12	106	0·57
	Mean for Group B_2		*104*	*0·55*
Group B_3	Fermented at pH 3·8 and left on the lees at pH 3·3	2	100	0·17
		4	102	0·20
		8	103	0·33
		12	107	0·23
	Mean for Group B_3		*103*	*0·23*
Group C	Fermented and left on the lees at pH 3·3	2	100	—
		4	104	T
		8	106	T
		12	106	T
	Mean for Group C		*104*	

— = no apparent growth.
T = slight turbidity, but no appreciable increase in titratable acidity.

It is also of interest to note that the bacterium developed quite readily and produced appreciable amounts of acid in wines which contained less total nitrogen than is present in most Australian fortified wines—cf. Table 9 with Table 12.

b. *The Effect of Temperature, pH, and Time of Racking.* In this experiment, a must which had a pH of 3·85 and a density of 14.0° Baumé was used. The must was divided into 36 one-litre portions in glass bottles, the necks of which were plugged with cotton-wool. The pH of 18 bottles of must was raised to 4·0 by the addition of potassium carbonate, and the pH of the other 18 bottles was lowered to 3·5 by the addition of tartaric acid. Ten c.c. of an active yeast culture in grape juice were added to each bottle. The bottles were then arranged in six groups and fermentation was carried out under the following conditions of pH and temperature:

I pH 3·5	A	temperature 15° C.
	B	temperature 25° C.
	C	temperature 35° C.
II pH 4·0	A	temperature 15° C.
	B	temperature 25° C.
	C	temperature 35° C.

When the density had been reduced to approximately 5° Baumé, the contents of each bottle were fortified with rectified spirit as in the previous experiment and the cotton-wool plugs were replaced by waxed corks to prevent undue loss of alcohol through evaporation. The bottles were then held at the above temperatures until they were racked.

Within each of the six groups, duplicate wines were racked and filtered through a Chamberland porcelain filter at intervals of one week, three weeks, and six weeks after fortification. The alcohol content and the acidity of each wine were adjusted to 20 per cent. by volume and pH 4·1 respectively, after the wines had been filtered.

The total nitrogen content of each wine was then determined by the micro-Kjeldahl method and samples were tested for susceptibility to bacterial attack as in the previous experiment.

TABLE 10

The Effects of Temperature, pH of the Must, and Time of Racking on the Susceptibility of Wines to Bacterial Spoilage.

	Treatment during manufacture.				Increase in titratable acidity after inoculating at pH 4·1 and incubating at 23°C. for 12 weeks (as grm./l tartaric acid).
	pH.	Temp. °C.	Time on gross lees (weeks).	Total N (mgm./l). Means.	Means.
I A	3·5	15	1	118	—
	"	"	3	129	—
	"	"	6	134	—
				Mean 127	—
B	3·5	25	1	128	—
	"	"	3	137	T
	"	"	6	135	0·83
				Mean 133	*0·28*
C	3·5	35	1	140	0·30
	"	"	3	146	0·95
	"	"	6	150	1·37
				Mean 145	*0·87*
II A	4·0	15	1	122	—
	"	"	3	145	—
	"	"	6	160	—
				Mean 142	—
B	4·0	25	1	151	—
	"	"	3	163	T
	"	"	6	164	1·10
				Mean 159	*0·37*
C	4·0	35	1	160	0·80
	"	"	3	175	1·08
	"	"	6	170	1·97
				Mean 168	*1·28*

— = no apparent growth.
T = slight turbidity produced, but no appreciable change in titratable acidity.

The effects of each of the three factors, pH, temperature, and time of racking, are shown in Table 10. The effect of temperature on the total nitrogen content of the wines and

also on the susceptibility of the wines to bacterial attack was very pronounced, and the effect of pH was again in evidence. The time of racking had a greater effect in this experiment than in the previous one. This is probably due, in part, to the fact that the newly racked wines were thoroughly clarified by means of a porcelain filter in the second experiment, whereas the wines of the first experiment were merely decanted and filtered through Whatman No. 2 filter paper.

TABLE 11

Effect of Yeast Autolysate on Growth of Lactobacillus *Type I in Wine. 12 weeks at 23° C.*

pH.	Yeast autolysate added, per cent.	Increased titratable acidity (as grm./l tartaric acid).
Adjusted to 4·3	nil	No growth
	0·5	0·32
	1·0	0·83
	2·0	1·95

The experimental results presented in Tables 9 and 10 indicate that the conditions of manufacture and the acidity of the must have a very important effect on the susceptibility of the wine to bacterial attack. From the same musts, or musts differing from one another only as regards acidity, it is possible to make either susceptible or non-susceptible wines by varying the conditions of manufacture. The bacterium *(Lactobacillus* Type I) failed to grow in samples of the original grape juices used in the above experiments, even after pH adjustment, although the grape juices were, of course, much richer in ordinary nutrients than the resulting wines. The susceptibility of these wines would therefore appear to be due to the liberation of some activating factor or to the removal of some inhibitory factor during manufacture. It is significant, however, that the conditions which led to the production of susceptible wines were high temperature and high pH during the period that the wines were in contact with the yeast cells, and also prolongation of this period, for these are just the conditions which are known to favour the autolysis of yeast cells. Moreover, samples of the non-susceptible wines which were made in the

above experiments supported a vigorous growth of the bacterium when small amounts of yeast autolysate were added and the pH adjusted prior to inoculation. The example given in Table 11 is typical of results obtained from adding yeast autolysate to these wines prior to inoculation.

4. Nitrogen Content of Wines

Much of the nitrogenous matter present in must is assimilated by the yeast during alcoholic fermentation. A normal wine is thus a poorer culture medium, as regards nitrogenous nutrients, than the must from which it was made. Laborde (1898) found that both ammonia and organic nitrogen were removed from the liquid during alcoholic fermentation. During the final stages of fermentation, however, organic nitrogen was slowly liberated from the yeast cells into the wine. This increase in the organic nitrogen content of the wine was found to proceed more rapidly at 36° C. than at lower temperatures. According to Niehaus (1938), 50 to 58 per cent. of the total nitrogen in must is removed during the first two days of alcoholic fermentation, and this result is not greatly affected by the strain of yeast used for the fermentation. Niehaus also found that the total nitrogen content of South African musts was not appreciably affected by the application of different fertilizers to the vineyards nor by abnormal ripening caused by drought. However, the total nitrogen content of young South African wines was found to increase during long contact with the gross lees.

Manceau (1903, 1906) noticed that wines made from grapes which had been attacked by Downy Mildew *(Plasmopara viticola)* contained abnormally large amounts of ammonia and total nitrogen. The relative frequency with which such wines are attacked by spoilage bacteria has been attributed to their high nitrogen content by Ventre (1931).

Genevois and Ribéreau-Gayon (1935) found no relationship between the amount of total- or amino-nitrogen and the type of wine or the country of origin, but they record that the nitrogen content of the must and the wine increases during successive stages of pressing. Benvegnin and Capt (1936)

also found that the amount of organic nitrogen in the must increased during successive stages of pressing.

Muth and Malsch (1934) determined different fractions of nitrogen in musts and wines and found that ammonia and protein represented quite small fractions of the total nitrogen present. By far the greater portion of the nitrogen in wines was in the form of amino acids and substances of intermediate complexity, corresponding to peptides and peptones, which were precipitated by phosphotungstic acid, but not by 80 per cent. alcohol and sodium sulphate.

In order to investigate the relationship between the incidence of bacterial spoilage and the nitrogen content of Australian fortified wines, a number of sound and unsound wines were analysed for different nitrogen fractions as follows:

Total nitrogen—Micro-Kjeldahl method.

Protein nitrogen—Total nitrogen in tungstic acid precipitate.

Amino-nitrogen—Method of Van Slyke.

Ammonia—Ammonia liberated with milk of lime and distilled over under reduced pressure, at 40° C., into standard H_2SO_4.

The figures set out in Table 12 are typical of the analyses of a number of wines. The undetermined fraction shown in column 6 of the table represents the difference between the total nitrogen and the sum of the three fractions determined, and corresponds, to some extent, with the phosphotungstic acid precipitated fraction of Muth and Malsch.

Of the unsound wines listed in the table, only number 37 and number 25 had undergone any very marked bacterial spoilage at the time the analyses were carried out. In general, the unsound wines contain more total nitrogen, and also more of each of the fractions determined, than the sound wines. Many exceptions have been noticed, however, and no clear relationship was noticed between the incidence of bacterial spoilage and the nitrogen content of the wines.

The total-nitrogen contents of fortified wines which were made in the laboratory were included in Tables 9 and 10. As has already been pointed out, bacteria grew quite vigorously in several of these wines, which contained less than 150 mgm.

of total-nitrogen per litre. Only four out of fifty commercial wines which were analysed contained less than 150 mgm. of total-nitrogen per litre. It therefore seems very unlikely that the total-nitrogen content of commercial wines is often insufficient for vigorous bacterial growth.

TABLE 12

Nitrogen Content of Sound and Unsound Wines.

		Nitrogen in mgm. per litre.				
	Type.	Protein N.	Ammonia N.	Amino N.	Undetermined.	Total N.
Sound wines.						
No. 8	Sweet red	5	7	32	209	253
27	" "	7	6	65	216	294
26	Sweet white	18	6	69	187	280
29	" "	12	10	123	423	568
36	Muscat	14	7	34	159	214
Unsound wines.						
37	Sweet red	3	23	58	203	287
16	Sweet white	20	34	69	262	385
19	" "	33	15	60	257	365
22	" "	11	33	88	284	416
25	" "	21	9	94	270	394
33	" "	3	5	30	78	116
40	" "	14	12	101	297	424
34	Muscat	16	6	40	179	241
35	"	10	5	36	184	235

Orla-Jensen (1919, 1935) and Orla-Jensen, Otte, and Snog-Kjaer (1936) showed that the lactic acid bacteria are very fastidious in regard to their nitrogen requirements, and that a number of amino-acids are necessary for normal growth. Thus it would seem that growth of the bacteria in wines is more likely to be limited by lack of some particular amino-acid than by insufficiency of total-nitrogen. However, the nature of the nitrogenous constituents in wines has not been sufficiently defined by analyses to provide experimental data on this point.

The results of experiments which have been described in previous sections indicate that the essential nitrogenous constituents which may be lacking in some wines are supplied in

adequate amounts by the addition of autolysed yeast, or by the autolysis of yeast cells in the wine. The figures presented in Tables 9 and 10 show that within each experiment there was a relationship between the nitrogen content of the wine and the susceptibility of the wine to bacterial attack. It is possible therefore, that the substances which favoured bacterial growth in these wines included certain amino-acids as well as accessory growth substances, in the more usual meaning of the term. Since such amino-acids would be liberated in the wine during autolysis of the yeast cells, the conditions favouring their occurrence in wines would be identical with the conditions favouring the occurrence of the accessory growth substances. These conditions have already been discussed.

5. Presence of Fermentable Sugars or Organic Acids

None of the strains of bacteria studied is able to grow in the autolysed yeast medium unless fermentable sugars or organic acids are added. Reference has already been made to the failure of the bacteria to grow in dry wines, and it seems likely that lack of fermentable sugar is the factor which limits bacterial growth in these wines. Some bacterial growth has occurred in wines which contained less than 0·1 per cent. of sugar, but the production of lactic and acetic acids was not appreciable under these circumstances. The addition of malic or citric acid to a dry wine renders it a more favourable medium for the growth of strains which are able to attack these acids, provided that the pH of the wine is not too low. However, no samples of commercial wines, which have become spoiled as a result of bacterial decomposition of organic acids, have been encountered during the investigations.

Of the various carbon and energy sources which have been added to culture media, those which are attacked most vigorously by all strains of the bacteria are levulose and dextrose, the two principal sugars present in must and sweet wines. Wines of low alcohol content which contain appreciable amounts of residual sugar are often attacked by bacteria. Such wines need to be treated with special care, for their low alcohol content renders them liable to attack by types of bacteria which are not able to grow in fortified wines. Thus, the amount of

residual sugar present in wines of low alcohol content is an important factor in relation to bacterial spoilage, but in the case of fortified wines, variations in the amount of sugar present, within normal limits, have little effect on bacterial growth or acid production. In the laboratory, typical bacterial spoilage has been induced in fortified wines containing from 1 to 12 per cent. of reducing sugar.

6. Alcohol Content of Wines

The alcohol tolerance of the different types of bacteria under discussion has already been mentioned. Although some strains of the bacteria are able to grow in media containing 20 per cent. of alcohol, they do so slowly, and the inhibiting effect of alcohol is noticeable at much lower concentrations. The figures presented in Table 7 show the slowness of growth and acid production in the presence of high concentrations of alcohol. Thus at 23° C., a strain of Type V increased the titratable acidity of autolysed yeast medium by an amount equivalent to 2·9 grm. of tartaric acid per litre in 7 days. In the presence of 10 per cent. of alcohol, however, the increase in titratable acidity was only 0·53 grm. per litre in 7 days. Similarly, the titratable acidity of the medium which contained 15 per cent. of alcohol was increased by 1·6 grm. per litre in 28 days, but twice as much time was required to produce this amount of acid in the presence of 20 per cent. of alcohol.

The inhibiting effect of alcohol is most marked at high temperatures. High concentrations of alcohol in the medium thus lower the maximum temperature for growth, and also the optimum temperature as measured by the production of acid during the early stages of growth.

The alcohol content of wine is of great importance in relation to bacterial spoilage. Wines which contain 20 per cent. of alcohol are not immune from attack, but they are attacked by fewer types of bacteria than are wines containing 18 per cent. of alcohol. Practically all of the strains which have been found capable of growing vigorously in 20 per cent. of alcohol belong to Type I and are very exacting in their growth

requirements. It has already been pointed out that some strains of Types III, IV, and V which are less tolerant of alcohol than Type I are capable of growing in media which will not support the growth of Type I. Thus, many wines which would undergo bacterial spoilage while containing 18 to 19 per cent. of alcohol, might remain sound if fortified to a strength of 20 per cent.

7. Sulphurous Acid—SO_2*

According to Ventre (1931) bacterial spoilage is prevented by the presence of 50 parts per million of SO_2 in the wine. Arena (1936), on the other hand, found that some of the bacteria which cause spoilage in the wines of Argentina have a very high tolerance of SO_2. There seems little doubt, however, that the high tolerance recorded by Arena is accounted for by the fact that his experiments were carried out in a neutral medium, and consequently the bacteria were exposed to neutral sulphites rather than to free sulphurous acid and acid sulphites.

As has already been pointed out, most of the investigations of bacterial spoilage carried out in Europe have been concerned with 'dry' wines. This fact is of special significance with regard to the amounts of SO_2 recommended, for the behaviour of SO_2 is very greatly affected by the presence of appreciable amounts of reducing sugar. When SO_2 is added to a must or a sweet wine, a portion of it is bound in loose combination by the sugars, the proportion which is bound in this way depending on the amount of sugar present and on the amount of SO_2 added, as well as other factors. It is well known that 'bound SO_2' has a very much weaker antiseptic effect on yeasts than has 'free SO_2.' Thus a must which has been treated with sufficient SO_2 to prevent fermentation by a yeast introduced immediately, may ferment quite readily if

* According to common oenological usage, the term "SO_2" includes not only pure sulphur dioxide, but also aqueous solutions of H_2SO_3 and the acid sulphites present in musts and wines which have been treated with sulphur dioxide, sulphurous acid or potassium metabisulphite, for the concentrations of these are determined and expressed in parts per million of SO_2.

inoculated with the same yeast a few days later. In the latter case a considerable proportion of the SO_2 has passed into the 'bound' state during the interval between the addition of the SO_2 and the introduction of the yeast. Bioletti and Cruess (1912) sowed yeasts in musts to which different amounts of SO_2 had been added, and observed the time taken for fermentation to commence in the presence of different amounts of free and bound SO_2. Under these conditions, the antiseptic power of the free SO_2 was found to be 30 to 60 times as great as that of the bound SO_2.

It is commonly stated that bound SO_2 has very little antiseptic power, but such statements appear to be based entirely on the results of experiments carried out with yeasts. The relative antiseptic power of bound and free SO_2 towards the spoilage bacteria does not seem to have been thoroughly investigated.

In studying the effect of SO_2 on the bacteria causing spoilage of fortified wines, experiments have been carried out in order to obtain information concerning the following points in particular:

Amount of SO_2 necessary to check growth when added to actively growing cultures.

Amount of SO_2 necessary to prevent growth when added to the medium before inoculation.

Relative antiseptic effect of bound and free SO_2 on the bacteria.

Effectiveness of adding SO_2 to the must as a means of preventing bacterial growth in the resulting wine.

Amount of SO_2 necessary to kill the bacteria in different periods of time.

Strain differences: Occurrence of strains with a high SO_2 tolerance.

It has been pointed out by Collier (1938) and by Moreau and Vinet (1938) that the antiseptic power of SO_2 increases with increased hydrogen ion concentration. The experiments described in this section were carried out in media with a pH of 4·2. Although this figure represents a lower degree of acidity than that of most fortified wines, it is within the pH

range of these wines and also within the pH range which favours vigorous bacterial growth. The amount of SO_2 which is required to check bacterial growth under these conditions should be more than sufficient to check bacterial growth in wines which have a lower pH value.

(1) Amount of SO_2 Required to Check Growth when added to Actively Growing Cultures.

Six different strains of bacteria were sown in 4-ounce bottles of autolysed yeast medium. These cultures were then incubated at 23° C. for three days, by which time growth was evident in all cases. After the contents of each bottle had been thoroughly shaken, they were divided among sterile test-tubes, and 2 c.c. of an aqueous solution of potassium metabisulphite were added to each tube. (The aqueous solutions of metabisulphite were of such strengths as to give the concentrations of SO_2 indicated in Table 13). The tubes were then incubated at 23° C. for a further 21 days, and at the end of this period the increased titratable acidity of the cultures was determined. The results of this experiment are presented in Table 13.

TABLE 13

Effect of SO_2 on the Growth of Lactobacillus *Type I, II and V.*

Organism.		Increase in titratable acidity (as g/l tartaric acid) in the presence of different amounts of SO_2.				
Type.	Strain.	Without SO_2.	20 p.p.m. SO_2.	40 p.p.m. SO_2.	80 p.p.m. SO_2.	100 p.p.m. SO_2.
I	L1	3·35	1·60	0·55	0·10	nil
	L5	4·60	3·50	0·35	nil	nil
II	L7a	3·40	3·45	2·30	nil	nil
	L7b	3·85	3·75	2·60	nil	nil
V	L6a	3·30	3·20	2·60	nil	nil
	L6b	3·80	3·65	3·25	0·10	nil

Although the different strains appear to differ somewhat in their sensitivity to small amounts of SO_2, they were all inhibited to a marked degree by 80 p.p.m., and all cultures which received 100 p.p.m. cleared within a few days, leaving a deposit in the bottom of the tube.

(2) Inhibitory Effect of 'Bound' and 'Free' SO_2 added to the Medium before Inoculation.

In the previous experiment the bacteria were already present in the medium at the time that the SO_2 was added. Thus a considerable proportion of the SO_2 would have been present in the free form at the time it came in contact with the bacteria. When SO_2 is added to sweet wines, a portion of it is soon bound by the sugars, so that if bacteria gain access to these wines at a later date they are exposed to both bound and free SO_2. In view of the relatively weak antiseptic effect of bound SO_2 on yeasts, it is important to know whether it has any appreciable effect on the spoilage bacteria.

The figures presented in Table 14 represent the results of an experiment designed to furnish information concerning the effectiveness of bound and free SO_2 in preventing the growth of different strains of the spoilage bacteria. In this experiment a susceptible sweet white wine was chosen as the culture medium.

The wine was sterilized by means of a Chamberland porcelain filter, and after filtration had the following characteristics: Alcohol content 18·4 per cent. by volume, pH 4·2, titratable acidity 4·65 g/l as tartaric acid, reducing sugar 7·6 per cent. as dextrose. The wine was placed in sterile 26-ounce bottles and different amounts of a 1·0 per cent. solution of potassium metabisulphite were added. The bottles were then corked securely and held at room temperature (16° to 18° C.). At intervals of ½ hour, 24 hours, 48 hours, and 7 days after the potassium metabisulphite had been added, samples (each of 180 c.c.) were withdrawn from each bottle. Each of the samples was treated in the following manner immediately after being withdrawn from the bottle: Total SO_2 and free SO_2 were determined by the Rippert method, and portions were placed in eleven sterile test-tubes. Duplicate tubes were then inoculated with five different strains of bacteria and were incubated at 23° C. for 28 days. At the end of this period the contents of the tubes were examined for bacterial growth and for increase in titratable acidity as compared with uninoculated controls.

TABLE 14

Inhibiting Effects of 'Bound' and 'Free' SO_2 towards Lactobacillus *Types I, II and V.*

		Bottle No.	1	2	3	4	5	6
A. After ½ hour.	Total SO_2 p.p.m.		nil	54	77	99	155	204
	Free SO_2 p.p.m.		nil	7	12	18	44	85
	Acid production by bacteria in 28 days							
	Type I	Strain L1	4·6	1·5	—	—	—	—
		L5	11·2	0·9	—	—	—	—
		L8	20·7	7·5	—	—	—	—
	Type II	L7	11·9	0·1	—	—	—	—
	Type V	L6	9·1	1·5	—	—	—	—
B. After 24 hours.	Total SO_2 p.p.m.		nil	54	77	99	149	191
	Free SO_2 p.p.m.		nil	4	8	10	23	44
	Acid production by bacteria in 28 days							
	Type I	Strain L1	5·5	1·0	—	—	—	—
		L5	13·8	0·5	—	—	—	—
		L8	21·3	10·6	—	—	—	—
	Type II	L7	11·5	0·2	—	—	—	—
	Type V	L6	11·2	2·9	—	—	—	—
C. After 48 hours.	Total SO_2 p.p.m.		nil	50	76	96	143	183
	Free SO_2 p.p.m.		nil	3	4	7	19	36
	Acid production by bacteria in 28 days							
	Type I	Strain L1	5·4	0·6	—	—	—	—
		L5	13·6	0·2	—	—	—	—
		L8	19·5	4·0	—	—	—	—
	Type II	L7	11·3	1·2	—	—	—	—
	Type V	L6	9·2	2·4	—	—	—	—
D. After 7 days.	Total SO_2 p.p.m.		nil	45	69	89	124	161
	Free SO_2 p.p.m.		nil	2	2	5	12	22
	Acid production by bacteria in 28 days							
	Type I	Strain L1	5·6	3·9	—	—	—	—
		L5	14·8	8·7	—	—	—	—
		L8	20·7	10·3	0·2	—	—	—
	Type II	L7	12·3	3·0	—	—	—	—
	Type V	L6	10·3	4·8	0·1	—	—	—

The results presented in Table 14 show the total and free SO_2 at the time that each series of inoculations was carried out, and also the amounts of acid produced by the different strains of bacteria under each set of conditions. It can be seen that a considerable portion of the SO_2 was bound by the sugars within

a short time. Even after half-an-hour only a small proportion of the SO_2 remained in the free state, and this amount had fallen still further at the end of a week.

It appears from the results of this experiment that the bound SO_2 has a considerable inhibiting effect on the bacteria under investigation. The bacteria failed to develop appreciably in the wines from bottles No. 3 and 4, even when sown seven days after the SO_2 had been added, and although the amount of free SO_2 in these wines at this stage was less than the amount of free SO_2 in No. 2 at the time that the first series of inoculations was made. Under the conditions of the experiment, approximately 75 to 80 parts per million of SO_2 was sufficient to prevent bacterial growth, although most of the SO_2 was present in the bound form at the time that the bacteria were introduced. Further data on this point are provided in the next experiment.

(3) Effect of adding SO_2 to the Must, on Susceptibility of the Resulting Wine.

A series of fortified white wines was made from a Doradillo must which had a density of 12·0° Bé, a pH of 3·8, and a titratable acidity of 3·4 grm. per litre as tartaric acid. One-litre portions of this must were placed in a number of glass bottles, and the pH and SO_2 content of duplicate portions were adjusted in accordance with the following scheme:

A pH 3·8
1. No SO_2 added.
2. Added sufficient 2 p.c. $K_2S_2O_5$ solution to bring SO_2 content to 150 p.p.m.
3. Added sufficient 2 p.c. $K_2S_2O_5$ solution to bring SO_2 content to 300 p.p.m.

B pH 4·2
1. No SO_2 added.
2. Added sufficient 2 p.c. $K_2S_2O_5$ solution to bring SO_2 content to 150 p.p.m.
3. Added sufficient 2 p.c. $K_2S_2O_5$ solution to bring SO_2 content to 300 p.p.m.

Fortified wines were made from each of these by the laboratory procedure previously described, and the wines were racked off the lees and filtered six weeks after fortification.

After the pH of all finished wines had been adjusted to 4·2 and the alcohol content had been adjusted to 20 per cent. by volume, the amounts of total and free SO_2 in each wine were determined. Samples of each wine were placed in sterile 4-ounce bottles and inoculated with *Lactobacillus* Type I, and the bottles, after being securely corked, were incubated at 23° C. At the end of eight weeks the inoculated samples were examined for bacterial growth and for increased titratable acidity as compared with uninoculated controls. Samples which showed no bacterial growth at the end of eight weeks were incubated for a further period of six weeks, but none of these showed bacterial growth at the end of the second period of incubation. The results presented in Table 15 represent the amounts of acid produced by the bacterium in the inoculated samples.

TABLE 15

Growth of Lactobacillus *Type I in Wines made from a must to which different amounts of SO_2 had been added.*

pH of must.	SO_2 added to must (p.p.m.).	SO_2 content of Wines. (mean of duplicate wines). Total	Free	Bacterial growth. Increased titratable acidity as g/l tartaric acid (mean of duplicate wines).
3·8	nil	—	–	3·2
3·8	150	70	4	T
3·8	300	172	7	No growth
4·2	nil	—	–	4·9
4·2	150	69	6	0·3
4·2	300	166	9	No growth

Note. pH of all samples when inoculated with bacteria was 4·2.
T = slight turbidity, but no appreciable increase in titratable acidity.

A considerable proportion of the SO_2 which was added to the must disappeared during fermentation and filtering, but sufficient remained in the wine to have a very marked effect on the growth of bacteria subsequently introduced. It is of interest to note that the amount of free SO_2 in the finished wines was quite low in all cases—less than 10 p.p.m. even in the wines made from the most heavily sulphited must. As has previously been mentioned, the bound SO_2 appears to have an

appreciable inhibitory effect on the growth of the bacteria under investigation.

In a previous section it was shown that the pH and temperature of the must during fermentation, and of the wine prior to racking, have an important effect on the susceptibility of the finished wine to bacterial attack. This effect is distinct from the direct effect of pH and temperature on bacterial growth, and appears to be related to the autolysis of yeast cells in the lees. It seemed desirable to ascertain whether SO_2 played a similar role, or whether the results presented in Table 15 are due entirely to the direct effect of SO_2 on the bacterium. Accordingly, portions of the wines made from the must which had received 300 p.p.m. of SO_2 were inoculated and incubated after being treated as follows:

(a) 1 per cent. of yeast-autolysate added.
(b) SO_2 content reduced to 30 p.p.m. by heating to 90° C. under reduced pressure. The wine was then made up to the original bulk with water and alcohol so as to contain 20 per cent. of alcohol by volume.

On incubation, it was found that no growth occurred in the wines which had received yeast-autolysate but which had not been desulphited. On the other hand, vigorous growth occurred in the samples of wine from which the SO_2 had been removed. Thus the failure of the bacterium to grow in the wines made from the heavily sulphited must appears to be due to the presence of SO_2 in these wines and not to the lack of essential growth substances.

(4) Amount of SO_2 required to Kill the Bacteria in a Sweet Wine.

Bacteria were grown in 4-ounce bottles of a susceptible sweet white wine which contained 3 per cent. of reducing sugar, 14 per cent. of alcohol, and had a pH of 4·2. The cultures were incubated at 23° C. for 12 days, by which time vigorous growth had occurred. One-c.c. portions of aqueous solutions of potassium metabisulphite (of appropriate strengths) were then added to the cultures and the bottles were securely corked and returned to the incubator. After different periods from the

time that the metabisulphite was added, duplicate tubes of a susceptible wine which contained 10 per cent. of alcohol were inoculated from each bottle; 0·5 c.c. of inoculum was used in each case. The tubes were incubated at 23° C. for 21 days and were then examined for bacterial growth.

The viability of *Lactobacillus* Type I, after being exposed to different amounts of SO_2 for different periods of time, is shown in Table 16. The results of a similar experiment with *Lactobacillus* Type V are shown in Table 17.

TABLE 16

Viability of Lactobacillus *Type I after Different Periods in Wine containing different amounts of* SO_2.

Bottle No.	SO_2 p.p.m.	Viability (growth on subculture) after diff. periods. ½ hour.	24 hours.	7 days.	14 days.	21 days.
1	nil	+ +	+ +	+ +	+ +	+ +
2	27	+ +	+ +	+ +	+ +	+ +
3	54	+ +	+ +	+ +	+ +	+ +
4	80	+ +	+ —	— —	— —	— —
5	106	+ +	— —	— —	— —	— —
6	159	+ +	— —	— —	— —	— —
7	212	+ +	— —	— —	— —	— —

+ = growth.
— = no growth.

TABLE 17

Viability of Lactobacillus *Type V after Different Periods in Wine containing different amounts of* SO_2.

Bottle No.	SO_2 p.p.m.	Viability (growth on subculture) after different periods. ½ hr.	1 hr.	6 hrs.	24 hrs.	48 hrs.	7 days.	14 days.	21 days.
1	nil	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
2	25	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
3	50	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
4	75	+ +	+ +	+ +	+ +	+ +	— —	— —	— —
5	100	+ +	+ +	+ +	+ +	— —	— —	— —	— —
6	150	+ +	+ +	+ +	+ +	— —	— —	— —	— —
7	200	+ +	+ +	+ +	+ +	— —	— —	— —	— —

+ = growth.
— = no growth.

Under these conditions both organisms were viable after three weeks in a medium treated with 50 parts per million of SO_2. Although the two types of bacteria differed in their viability after 24 hours in the heavily sulphited medium, they were both killed in 48 hours by 100 parts per million of SO_2. Such a result cannot be relied on under industrial conditions, however, for in many cases bacteria have been isolated from gross lees after the original must had been treated with 250 parts per million of SO_2. This apparent discrepancy between results obtained in the laboratory and those observed under industrial conditions is probably due to the uneven distribution of the SO_2 throughout the contents of the large tanks used in the wineries. Owing to the difficulty of mixing the added SO_2 evenly throughout a large volume of must, localized zones of low SO_2 concentration are likely to occur and bacteria may remain viable in such zones. Once fermentation becomes vigorous, the liquid tends to become stirred thoroughly, but the SO_2 content of the liquid falls quite rapidly at this stage.

(5) Strain Differences in Regard to SO_2 Tolerance.

Since it was not practicable to include more than a few strains of bacteria in the foregoing experiments, it seemed desirable to ascertain whether some strains had an appreciably higher tolerance towards SO_2 than the strains used in the experiments already described. Thirty different strains, representing all five types of bacteria, were sown in tubes of autolysed yeast medium which contained different amounts of SO_2. These tubes were incubated at 23° C. for 14 days and were then examined for growth. The results presented in Table 18 indicate the number of strains which grew in the presence of different amounts of SO_2.

Although some differences were noticed in the tolerance of different strains towards SO_2, there was no sign of growth in the medium to which 100 p.p.m. of SO_2 had been added, and three strains produced only weak growth in the presence of 75 p.p.m. None of the strains tested had an unduly high tolerance towards SO_2, and 100 parts per million appears to be adequate to prevent their growth in this medium. Somewhat lower concentrations of SO_2 are sufficient to check bacterial

growth in wine, however. In a sweet wine containing 18 per cent. of alcohol and having a pH of 4·2, none of the strains mentioned in Table 18 were able to grow in the presence of 80 p.p.m. of SO_2.

TABLE 18

Growth of Thirty Strains of Bacteria in medium containing different amounts of SO_2.

Type and No. of strains tested.	No. of strains which grew in presence of different amounts of SO_2. Failed to grow in 50 p.p.m. SO_2.	Grew in 50 p.p.m. but not in 75 p.p.m.	Grew in 75 p.p.m. but not in 100 p.p.m.	Grew in 100 p.p.m.
Type I 5 strains	2	2	1	–
II 4 "	–	4	–	–
III 2 "	1	1	–	–
IV 8 "	1	6	1	–
V 11 "	4	6	1	–

8. Tannin.

Tannin is used extensively in winemaking and is added both to musts and to wines in order to facilitate fining with gelatin subsequently, and also to improve the flavour and protect the colour of certain wines which contain insufficient tannin. It is often claimed that the tannin in wine has antiseptic properties, but the opinions on this subject which have been published by different authorities are rather conflicting.

Ventre (1930) observed that musts which have a high tannin content are often difficult to ferment, and he claimed that the tannin in these musts inhibits both the multiplication of yeast cells and alcoholic fermentation. A series of experiments which was carried out by Laborde is mentioned by Ventre (1931). Laborde found that a bacterium causing 'tourne' grew quite readily in a wine which contained 7 grm. of natural grape tannin per litre. Removal of different amounts of this tannin by fining with gelatin did not affect bacterial growth, and it was concluded that the natural tannin of wine had practically no effect on this organism. When commercial oak-gall tannin was added to wine, however, it was found that bacterial growth was definitely retarded by

0·5 grm. per litre, and was almost completely checked by 1·0 grm. per litre.

According to Cruess (1935b), the amounts of tannin which are normally added to musts and wines retard the growth of spoilage bacteria but have no effect on the growth of 'wild yeasts.' Recently Bertin (1938) claimed that the tannins used in wine-making have no antiseptic properties whatever, and that their only useful function is in the fining of wines. However, Bertin's conclusion appears to be based on data obtained from experiments which were carried out with yeasts and therefore does not necessarily apply to bacteria. It is a well-known fact that different organisms differ very considerably in their resistance to many antiseptics.

In studying the effect of tannin on the bacterial spoilage of fortified wines, the following points have been investigated experimentally:

Effect of normal concentrations of tannin on the growth of the spoilage bacteria.

Effectiveness of adding tannin to the must as a means of preventing bacterial growth in the finished wine.

The nature of the growth-inhibiting effect of tannin—precipitation of essential nutrients and accessory growth substances or direct effect on the bacteria.

Comparison of natural grape tannin with different commercial oenological tannins, as regards the effect on bacterial growth.

Throughout the following experiments the amounts of tannin in wines were determined by the A.O.A.C. official method. The available-tannin content of commercial samples was determined by permanganate titration and precipitation with gelatin and NaCl as in Hunt's modification of the Lowenthal method (Allen, 1927).

(1) Effect of Tannin on Actively Growing Cultures.

Duplicate cultures of three different strains of bacteria were grown in 4-ounce bottles of autolysed yeast medium. The cultures were incubated at 23° C. for three days, by which time growth was evident. Each culture was then divided between five sterile test-tubes and 2 c.c. of aqueous solutions of tannin were added to each tube. A high-grade,

commercial, oenological tannin, prepared from gall-nuts and containing 78 per cent. of available tannin was used. The aqueous solutions of tannin were of such strengths as to provide the concentrations of tannin which are indicated in Table 19, when 2 c.c. portions were added to the cultures. After the tannin had been added the cultures were incubated at 23° C. for a further 14 days and were then examined for growth and acid production. The results of this experiment are presented in Table 19.

TABLE 19

Effect of Tannin on Growth of Lactobacillus *Types I, II and V in Autolysed Yeast Medium. Increase in titratable acidity as g/l tartaric acid after addition of different amounts of Tannin.*

	No. tannin.	1·0 g/l tannin.	2·0 g/l tannin.	5·0 g/l tannin.	10·0 g/l tannin.
Type I	2·9	0·5	0·3	nil	nil
II	5·1	2·7	1·4	0·7	0·3
V	2·8	0·9	0·6	0·3	nil

In this experiment the tannin exerted a very marked inhibitory effect on the three bacteria, and this effect was quite evident when 1·0 grm. per litre of tannin was added. It appears, however, that different strains of bacteria differ appreciably in their tolerance towards tannin and also that quite large amounts of tannin are needed to check the development of bacteria completely, when they are already growing in the medium.

(2) Effect of Tannin when Added to the Medium Prior to Inoculation.

Different amounts of tannin were added to two series of tubes of autolysed yeast medium. The contents of one series of tubes were then filtered through a Seitz E.K. filter, while the contents of the other series were left unfiltered. In both series duplicate tubes representing each treatment were inoculated with each of the three strains of bacteria which were used in the previous experiment. The cultures were incubated at 23° C., and after 14 days they were examined and compared with one another by visual inspection. The effect of different amounts of tannin on the growth of the bacteria is shown in Table 20.

TABLE 20

Growth of Bacteria in Autolysed Yeast Medium to which different amounts of Tannin had been added.

A. Not filtered before inoculation:

Tannin added (g/l)		nil	0·5	1·0	2·0
Growth of *Lactobacillus*	Type I	+ + +	+	—	—
" " "	Type II	+ + +	+ + +	+	—
" " "	Type V	+ + +	+ + +	—	—

B. Filtered before inoculation:

Tannin added (g/l)		nil	1·0	2·0	4·0
Tannin present after filtering (g/l)		nil	0·2	0·5	1·3
Growth of *Lactobacillus*	Type I	+ + +	+ +	+	—
" " "	Type II	+ + +	+ + +	+ +	—
" " "	Type V	+ + +	+ + +	+ +	—

— = no growth, + = weak growth, + + = strong growth,
+ + + = very strong growth.

Under these conditions the bacteria were unable to grow vigorously when the medium contained approximately 1 grm. per litre of tannin at the time of inoculation. Filtering the medium prior to inoculation removed a considerable portion of the tannin together with other precipitated material, and rendered the medium more favourable for bacterial growth. A comparison of the results of the filtered series with the results of the unfiltered series appears to indicate that it is the amount of tannin actually present in the medium, rather than the amount which has been used in previous treatment of the medium, which is of importance in preventing the growth of bacteria. Further data on this point are provided in subsequent experiments.

(3) Addition of Tannin to the Must—Effect on Susceptibility of the Finished Wine to Bacterial Attack.

A series of fortified wines was made from a Doradillo must which had a density of 12·0° Bé, a pH of 3·8, a titratable acidity of 3·4 grm. per litre (as tartaric acid), and a tannin content of 0·07 grm. per litre. One-litre portions of this must were placed in a number of glass bottles and the acidity and

tannin content of duplicate portions were adjusted in accordance with the following scheme:

A pH 3·8
1. No tannin added.
2. 0·5 grm. tannin added per litre.
3. 1·0 grm. tannin added per litre.

B pH 4·2
1. No tannin added.
2. 0·5 grm. tannin added per litre.
3. 1·0 grm. tannin added per litre.

The contents of each of the bottles were fermented at 25° C. and made into fortified wine by the laboratory procedure described in a previous section. The wines were fortified to a strength of 20 per cent. by volume of alcohol and were racked off the lees and filtered six weeks after fortification. After filtration, the alcohol content and acidity of all the finished wines were readjusted to 20 per cent. by volume and pH 4·2 respectively.

The tannin content of each wine was then determined and samples were placed in sterile 4-ounce bottles, inoculated with *Lactobacillus* Type I and incubated at 23° C. The inoculated samples were examined for bacterial growth from time to time, and after eight weeks the increase in titratable acidity, as compared with uninoculated controls, was determined. Samples which showed no bacterial growth at the end of eight weeks were incubated for a further six weeks, but none of these showed bacterial growth at the end of the second period of incubation.

In addition to the samples already mentioned, further portions of the wines were inoculated after being treated in the following manner:

i. The wines made from the must to which no tannin had been added (A1 and B1) received tannin at the rate of 0·5 grm. per litre.

ii. The wines made from must to which 1·0 grm. per litre of tannin had been added (A3 and B3) were fined with gelatin at the rate of 1 grm. per litre and filtered. In this way the tannin content of these wines was reduced to approximately 0·2 grm. per litre.

Details of the different treatments and the amounts of acid produced by the bacterium in the inoculated samples are shown in Table 21.

TABLE 21

Growth of Lactobacillus *Type I in Wines made from Must to which Different Amounts of Tannin had been added.*

Wine No.	pH of must.	Treatment. Addition of tannin.	Tannin content of wine (g/l).	Bacterial growth. Increased titratable acidity (g/l tartaric acid).
1	3·8	Nil	·07	2·2
2	3·8	Nil	·07	2·0
3	3·8	0·5 g/l added to the must	·37	1·0
4	3·8	" " " " "	·38	1·1
5	3·8	1·0 g/l added to the must	·68	No growth
6	3·8	" " " " "	·70	No growth
7	4·2	Nil	·06	3·6
8	4·2	Nil	·07	4·5
9	4·2	0·5 g/l added to the must	·43	2·5
10	4·2	" " " " "	·42	2·5
11	4·2	1·0 g/l added to the must	·78	No growth
12	4·2	" " " " "	·75	No growth
1a	3·8	0·5 g/l of tannin added to the wine immediately before inoculation.		No growth
2a	3·8			No growth
5a	3·8	tannin content of wine reduced to 0·2 g/l by fining with gelatin.		2·3
6a	3·8			1·7
7a	4·2	0·5 g/l of tannin added to the wine immediately before inoculation.		No growth
8a	4·2			No growth
11a	4·2	tannin content of wine reduced to 0·2 g/l by fining with gelatin.		3·5
12a	4·2			4·3

Note: pH of all wines when inoculated was 4·2.

Bacterial growth was very vigorous in the wines which were made without the addition of tannin either before or after the primary fermentation. Of these wines, the ones made from the must with the higher pH were the more af-

fected, but quite vigorous growth also occurred in the wines made from must with a pH of 3·8 without the addition of tannin. These results are in agreement with those of previous experiments.

Addition of tannin to the must at the rate of 0·5 grm. per litre reduced bacterial growth appreciably, particularly in the wines made from the more acid must, but this amount of tannin did not check growth completely. However, the bacterium failed to grow at all in the wines made from must which had received 1 grm. per litre of tannin.

The effect of tannin on bacterial growth as shown by the results in Table 21, appears to be due to the direct effect of tannin on the bacterium, rather than to any lack of essential growth substances, resulting from the presence of tannin during the primary fermentation. Wines 5, 6, 11, and 12, which were made from must which had received tannin at the rate of 1 grm. per litre, failed to support bacterial growth. When most of this added tannin had been removed by fining with gelatin, however, the bacterium grew quite vigorously (*vide* Wines 5a, 6a, 11a, and 12a). Moreover, the failure of the bacterium to grow in wines 5, 6, 11, and 12 is readily accounted for by the presence of approximately 0·7 grm. per litre of tannin in these wines, for the addition of 0·5 grm. per litre of tannin immediately before inoculation prevented bacterial growth in wines 1a, 2a, 7a, and 8a. Further information on this point is provided in the following experiment.

(4) Nature of the Effect Produced by Tannin—Precipitation of Essential Nutrients or Direct Effect on the Bacteria.

Duplicate portions of a sweet white wine were treated in the following manner:

1. Added 2 per cent. of yeast autolysate.
2. Added 2 per cent. of yeast autolysate and 1 grm. per litre of tannin.
3. Added 2 per cent. of yeast autolysate, 1 grm. per litre of tannin, and 2 grm. per litre of gelatin.

4. Added 2 per cent. of yeast autolysate and 2 grm. per litre of gelatin.
5. Added 2 grm. per litre of gelatin.
6. No addition.

The treated wines were all filtered, and the pH and alcohol content were adjusted to 4·2 and 20 per cent. by volume respectively. The wines were then placed in sterile 4-ounce bottles, inoculated with *Lactobacillus* Type I and incubated at 23° C. After ten weeks these wines were examined for bacterial growth and for increase in titratable acidity. The results are presented in Table 22.

TABLE 22

Effect of Tannin on the Growth of Lactobacillus *Type I in a Sweet White Wine.*

No.	Treatment.	Bacterial growth. Increased titratable acidity. (g/l tartaric acid).
1a 1b	2% yeast autolysate	4·75 4·8
2a 2b	2% yeast autolysate and 1 g/l of tannin	No growth No growth
3a 3b	2% yeast autolysate, 1 g/l of tannin and 2 g/l of gelatin	4·5 4·4
4a 4b	2% yeast autolysate and 2 g/l of gelatin	4·6 4·7
5a 5b	2 g/l of gelatin	No growth No growth
6a 6b	no addition	No growth No growth

Addition of yeast autolysate to the wine enabled the bacterium to grow very vigorously. Growth was checked completely by the addition of tannin at the rate of 1 grm. per litre, but when this tannin was removed from the wine by fining with gelatin, the bacterium grew almost as vigorously as in the wine which had received yeast autolysate only. It appears, therefore, that the failure of the bacterium to grow in wines 2a and 2b is due to the inhibitory effect of the tannin

and not to precipitation of nutrients or accessory growth substances as a result of treatment with tannin. The effect of gelatin in promoting growth in wines 3a and 3b appears to be due simply to the removal of tannin from these wines, for gelatin itself did not increase bacterial growth in the wine—compare wines 4a and 4b with 1a and 1b, and also wines 5a and 5b with 6a and 6b.

(5) Comparison of Different Tannins as Inhibitors of Bacterial Growth.

Several different tannins have been compared as regards their effect on bacterial growth. The commercial tannin used in the previous experiments was compared with other commercial tannins and also with natural grape tannin.

The results presented in Table 23 show the effects of pure tannic acid (Merck's analytical reagent) and of five different commercial tannins on bacterial growth in wine. The wine which was used in this experiment was a sweet white wine containing 20 per cent. by volume of alcohol and having a pH of 4·2. One hundred-c.c. portions of this wine were placed in each of twenty-six 4-ounce bottles and aqueous solutions of the tannins were added to twenty-four of these. The tannin solutions were made up in such a way that 1 c.c. contained 0·05 grm. of 'available tannin' in each case. As the tannins differed from one another in purity, the amounts of the commercial tannins were different in the different solutions. Each of the tannins was added to the wine in two different amounts and duplicate bottles received each treatment. After the tannins had been added, all bottles were inoculated with *Lactobacillus* Type I and were incubated at 23° C. They were examined for bacterial growth and for increased titratable acidity at the end of 10 weeks.

The amount of tannin required to prevent bacterial growth in this wine was approximately 0·5 grm. per litre, irrespective of which tannin was used, but the amount of commercial tannin needed to provide 0·5 grm. of 'available tannin' differed considerably from one tannin to another. This result appears to indicate that the inhibitory effect on the bacterium is associated

TABLE 23

Effect of different Tannins on the Growth of Lactobacillus *Type I in a Sweet White Wine.*

Tannin.	Particulars of tannin. Source.	Colour with ferric acetate.	Available tannin content (%).	Available tannin added to wine (g/l).	Bacterial growth. Increased titratable acidity (g/l tartaric acid).
1. Tannic acid	Merck's analytical reagent	Blue	96	0·5	T
				1·0	No growth
2. Commercial I	Oak galls	Blue	78	0·5	T
				1·0	No growth
3. Commercial II	Oak galls, Alcohol and water extraction	Blue	69	0·5	T (?)
				1·0	No growth
4. Commercial III	Oak galls, Water extraction	Blue	50	0·5	T
				1·0	No growth
5. Commercial IV	?	Blue	52	0·5	T
				1·0	No growth
6. Commercial V	?	Green	28	0·5	0·6
				1·0	No growth
No tannin added—controls				—	3·6

T = slight bacterial growth but no appreciable change in titratable acidity.

with the tannin itself rather than with any of the impurities, which are present in quite large amounts in some of the commercial products.

In another experiment natural grape tannin was compared with commercial tannin I as an inhibitor of bacterial growth in wine. In the absence of authentic supplies of pure grape tannin, a wine made from Shiraz pressings was used as a source of grape tannin. A portion of this wine was concentrated under reduced pressure and added to the remainder, thus raising the tannin content of the wine to 2·35 grm. per litre, and lowering the alcohol content to 15 per cent. by volume. The pH was adjusted to 4·2, and 1·0 per cent. of yeast autolysate was added to the wine in order to ensure that bacterial growth would not be limited by acidity or lack of essential growth substances. The wine was divided into three portions, two of which were fined with gelatin to reduce the tannin content to 1·3 and 1·8 grm. per litre respectively. These three portions were then subdivided, and different amounts of commercial tannin were added. Samples receiving each treatment were then inoculated with bacteria and were incubated at 23° C. for six weeks.

Details of the different treatments and the increase in titratable acidity brought about by two different bacteria are recorded in Table 24. The amount of grape tannin present in the wine was determined by the A.O.A.C. official method, using animal charcoal to precipitate tannin, and also by precipitation of the tannin with gelatin and NaCl as in Hunt's modification of the Lowenthal method (Allen, 1927). The A.O.A.C. official method is widely used for determining tannin in wines, but is listed as a method for determining 'tannin and colouring matter.' In many wines the results obtained by these two methods agree fairly closely, but in very highly-coloured wines, such as the material used in this experiment, the amount of tannin and colouring matter precipitated by animal charcoal is considerably greater than the amount precipitated by gelatin.

It appears from the results shown in Table 24 that natural grape tannin does inhibit the growth of bacteria in wine to

some extent, but that it is less effective in this respect than commercial oak tannin.

TABLE 24

Effect of Natural Grape Tannin and Commercial Oak Tannin on the Growth of Lactobacillus *Type I and Type V in Wine.*

Wine No.	Natural grape tannin (g/l). A.O.A.C. (official method).	Natural grape tannin (g/l). Precipitated by gelatin.	Added oak tannin g/l.	Bacterial growth. Increased titratable acidity. Type I	Bacterial growth. Increased titratable acidity. Type V (L36).
1	2·35	1·3	nil	No growth	5·0
2	"	"	0·2	"	4·3
3	"	"	0·4	"	3·2
4	"	"	0·7	"	1·2
5	1·8	0·9	nil	T	6·5
6	"	"	0·2	No growth	5·2
7	"	"	0·4	"	3·9
8	"	"	0·7	"	1·4
9	1·3	0·6	nil	6·0	6·9
10	"	"	0·2	T	5·5
11	"	"	0·4	No growth	4·6
12	"	"	0·7	"	2·8

The comparative ineffectiveness of natural grape tannin (as determined by the A.O.A.C. method) in preventing bacterial growth is probably accounted for by the fact that a considerable proportion of the material which is precipitated from pressings by animal charcoal, and is oxidizable by permanganate, is colouring matter rather than tannin.

The figures shown in the last column of Table 24 represent the acid produced by *Lactobacillus* Type V, strain L36. This strain was isolated from the lees of a sweet red wine. It has a relatively high tolerance of tannin, but has consistently failed to grow in 18 per cent. of alcohol and is not capable of growing vigorously in fortified wines.

CHAPTER IV

HEAT RESISTANCE OF THE BACTERIA

Wines are frequently pasteurized in order to destroy bacteria, but different authorities recommend different temperatures for this purpose. The bacteria studied by Gayon and Dubourg (1901) were destroyed by pasteurizing the wine at 60° C. for one minute. Semichon (1905) recommends that wines which contain 9 to 13 per cent. of alcohol and 4 to 6 grm. per litre of tartaric acid should be pasteurized for one minute at a temperature between 55° and 65° C., depending on the type of bacterium present. According to Ventre (1931) the temperature at which spoilage bacteria are destroyed in wines, in one minute, varies from 55° to 65° C., according to the alcohol content and the acidity of the wines. On the other hand, Cruess (1935c) found that it was necessary to heat certain Californian wines to 85° C. for one minute in order to destroy spoilage bacteria.

In the present investigation, the heat-resistance of the bacteria has been determined by a modification of the open tube method. The bacteria were heated in autolysed yeast medium at pH 4·3 and also in susceptible wine containing 15 per cent. of alcohol and having a pH of 4·2. Plugged test-tubes, each containing 10 c.c. of culture medium, were heated to the desired temperature in a water bath, and were then inoculated with 0·1 c.c. of a vigorous culture which had been grown in a similar medium. The temperature of the water bath was kept constant, and at the end of different periods after inoculation, tubes were withdrawn from the water bath and cooled rapidly in iced water. All tubes were then incubated at 23° C. for four weeks and were examined for bacterial growth at intervals. The temperatures to which the inoculated tubes were heated and also the periods of exposure to each of these temperatures are shown in Table 25. Four tubes were subjected to each treatment.

The heat-resistance of one strain of Type I and one strain of Type V in autolysed yeast medium and also in wine, was determined by the method indicated. The treatments which killed these two strains were then applied to eight other strains to determine whether any of these latter were more resistant to heat.

TABLE 25

Heat-resistance of Lactobacillus *Type I (Strain L1) and Type V (Strain L36) in Autolysed Yeast Medium and in Sweet White Wine containing 15 per cent. of Alcohol by Volume.*

Results = number of tubes in which growth occurred after incubating at 23° C. for 28 days. 4 tubes subjected to each treatment.

Medium.	Organism.	Temp. °C.	Duration of treatment. 1 min.	5 min.	10 min.	15 min.	20 min.
Autolysed yeast medium	Type I	55	4	4	4	4	2
		60	4	4	4	3	0
		65	3	2	3	1	0
		70	2	0	0	–	–
		75	0	0	–	–	–
	Type V	55	4	4	4	2	2
		60	4	4	1	1	0
		65	4	1	0	0	0
		70	0	0	0	–	–
Wine	Type I	55	4	4	4	1	0
		60	4	4	4	1	0
		65	3	3	2	0	0
		70	0	0	0	–	–
	Type V	55	4	3	4	2	0
		60	4	1	0	0	0
		65	2	0	0	–	–
		70	0	0	–	–	–

– = not determined.

The results, which are presented in Table 25, indicate that Type I is rather more resistant to heat than Type V. Both organisms, however, were killed within one minute at 75° C., or five minutes at 70° C., in the autolysed yeast medium, and within one minute at 70° C., or fifteen minutes at 65° C., in the wine.

Eight additional strains of bacteria were subjected to heat treatment as follows:

(a) In autolysed yeast medium at 70° C. for 1 minute and 5 minutes, and at 75° C. for 1 minute and 5 minutes.

(b) In wine containing 15 per cent. alcohol—at 70° C. for 1 minute and 5 minutes, and at 65° C. for 15 minutes and 20 minutes.

These tests were carried out in duplicate instead of quadruplicate, and the eight strains comprised one of Type I, one of Type V, and two of each of the other three types. None of these eight strains grew after heating for one minute at 75° C., or 5 minutes at 70° C. in autolysed yeast medium, or after heating for one minute at 70° C. or 15 minutes at 65° C. in the wine.

From the foregoing results, it appears that fortified wines may be pasteurized effectively by heating to 70° C. for one minute. Lower temperatures are likely to be ineffective in some cases, unless the period of treatment is prolonged.

The majority of fortified wines contain more alcohol and have a lower pH than the wine used in the foregoing experiments, and, consequently, they could be pasteurized effectively at somewhat lower temperatures. It is likely that the bacteria under investigation would be killed in some wines by pasteurization for one minute at temperatures between 55° and 65° C., as recommended by European authorities. A more severe treatment is evidently necessary to kill the bacteria in wines with a high pH, however, and these are the wines which are the most likely to require pasteurization.

CHAPTER V

CONTROL OF ACIDITY

THE results obtained in the experiments which have been described in the preceding pages indicate that the susceptibility of wine to bacterial spoilage depends very largely on the acidity of the must and of the resulting wine, and thus control of acidity may be expected to play an important part in control of spoilage.

The acidity of must or wine may be expressed either in pH values or in terms of titratable acidity, and although the pH value is the more reliable indication of the effect of acidity on bacterial growth and enzyme activity, it is not a reliable indication of sourness or acidity as registered by the human palate. Dietzel (1926) found that in mixtures of organic acids and their salts, sourness depends not only on the concentration of hydrogen ions, but also on the amounts of undissociated acid and acid anions present. This view has been borne out by the experience of the present writer. Wines which have approximately the same titratable acidity but which differ in pH, can be graded in order of pH by taste, but in a series of wines which differ in both titratable acidity and in pH, sourness appears to be more closely related to titratable acidity than to pH.

The palatability of the wine is, of course, one of the most important factors to be considered in advocating control measures. Thus, the problem of controlling acidity as a means of controlling bacterial spoilage, resolves itself into one of lowering the pH of the must and the wine without unduly increasing the titratable acidity of the wine. For adjusting acidity in this country, tartaric or citric acid is commonly added either to the must or the wine, or sometimes to both the must and the wine. It has already been pointed out that low pH values inhibit the autolysis of yeast cells in the gross lees, and for this reason the acid should be added to the

must rather than to the wine. As an example of the effects of adding tartaric and citric acids to must, the following experimental results are presented:

Tartaric and citric acids were added to different 400 c.c. portions of a Doradillo must and these were then made into fortified wines. After fortification the wines were held at 15° C. for 28 days and then the pH and titratable acidity of each were determined. The pH values and the titratable acidity of the different portions of must and the finished wines are shown in Table 26.

As can be seen, tartaric acid was more effective than citric acid in lowering the pH value without unduly increasing the titratable acidity (lines c and f). It is to be expected that tartaric acid would be more effective than citric acid in this respect in view of the greater degree of dissociation of tartaric acid, but the effect is accentuated by the precipitation of potassium hydrogen tartrate during fermentation and following fortification. From the figures in line g of Table 26, it appears that nearly all of the added citric acid remained in the wine, whereas nearly half of the added tartaric acid was precipitated as potassium hydrogen tartrate. It can be seen that although this precipitation lowers the titratable acidity considerably, it affects the pH only slightly as compared with the effect of adding an equivalent amount of tartaric acid to the must (compare lines c and f with i). This appears to be due to the difference in the degree of dissociation of the two acidic hydrogens of tartaric acid. As the acidic hydrogen of potassium hydrogen tartrate is only weakly dissociated, the precipitation of this salt removes only small amounts of hydrogen ions. Furthermore, the dissociation of tartaric acid is depressed in the presence of potassium hydrogen tartrate, so that removal of the latter tends to increase the dissociation of tartaric acid. The importance of the relationship between tartaric acid and potassium hydrogen tartrate has also been stressed by Brémond (1937), who found a close relationship between the ratio $\frac{\text{total tartrates}}{\text{total potash}}$ and the pH value of wines. Thus the high pH value of certain wines may be as much due to excess of potash

TABLE 26

pH and Titratable Acidity of Fortified Wines made from Must acidified with Citric and Tartaric Acids.

(Titratable acidity expressed as grms./litre tartaric acid).

Additions to the Must, expressed as g/l tartaric acid.	Nil.	Tartaric acid added. 1·2 g/l.	Tartaric acid added. 2·4 g/l.	Citric acid added. 1·25 g/l.	Citric acid added. 2·45 g/l.
I *Acidity of Must*					
a. Titratable acidity	4·55	5·75	6·95	5·80	7·00
b. pH	3·78	3·58	3·40	3·64	3·54
c. Decrease in pH/grm. increase in titratable acidity		0·17	0·16	0·11	0·10
II *Acidity of Wine*					
d. Titratable acidity	3·52	4·20	4·82	4·72	5·73
e. pH	3·85	3·64	3·45	3·71	3·57
f. Decrease in pH/grm. increase in titratable acidity		0·31	0·31	0·12	0·13
III *Loss of acidity during experiment*					
g. Fall in titratable acidity = a–d	1·03	1·55	2·13	1·08	1·27
h. Rise in pH = e–b	0·07	0·06	0·05	0·07	0·03
i. Rise in pH per grm. fall in titratable acidity = h/g	0·07	0·04	0·02	0·06	0·02

as to deficiency of tartaric acid in the must. When tartaric acid is added to such a must it not only increases the amount of acid present, but also serves as a vehicle for removing the excess potash.

TABLE 27

The pH and Titratable Acidity of several varieties of Grapes at different stages of ripeness.

1937 Season.

Locality.	Variety.	Date.	Density °Bé.	pH.	Titratable acidity g/l tartaric acid.
A	Shiraz	20th March	15·0	3·72	5·87
	"	1st April	16·0	3·88	4·80
	"	15th April	16·5	4·00	4·05
	Grenache	8th April	15·5	3·60	5·05
	"	15th April	16·0	3·67	4·92
	Mataro	1st April	14·2	3·70	5·50
	"	9th April	15·2	3·79	5·09
	"	29th April	15·3	3·86	4·72
	Gordo Blanco	1st April	13·6	3·76	5·00
	" "	15th April	13·8	3·77	4·96
	" "	29th April	14·2	3·81	4·80
	Doradillo	15th April	12·1	3·72	3·74
	"	21st April	12·4	3·76	3·62
	"	29th April	12·7	3·82	3·46
B	Doradillo	4th May	11·2	3·79	3·97
	"	22nd May	11·6	3·89	3·50
	"	3rd June	12·7	4·18	3·06
C	Doradillo	6th May	12·5	3·92	3·40
	"	16th May	13·2	3·97	3·05
	"	30th May	13·3	4·01	3·00

Although the addition of acid is an effective means of lowering the pH value of must, it is sometimes rather costly since some of our musts have pH values as high as 4·3. It appears, however, that such high pH values are often due to over-ripening of the grapes. According to Genevois (1938), the acidity of the grape falls continually during ripening, owing to the disappearance of organic acids and the accumulation of potassium, calcium, and magnesium. Thus if the grapes are allowed to remain on the vine until very late in the season the pH of the must is likely to be high.

The figures presented in Table 27 are taken from the results of a series of determinations carried out on the musts of several 'sweet wine varieties' grown in different districts in 1937. Although no general correlation was noticed between the density and the acidity of the musts, a fairly close correlation was noticed between the density and acidity of the must from any one variety grown in any one locality.

The figures for the variety Doradillo are particularly interesting, for in localities B and C this variety reached a satisfactory density only late in the season, when the pH value had become very high.

It is not suggested that high Baumé readings are always accompanied by high pH values, but when high Baumé readings can be obtained only by allowing the grapes to remain on the vine until very late in the season, then the pH of the must is likely to be high.

SUMMARY

THE spoilage of Australian fortified wines, which is characterized by turbidity and an increase in both volatile and fixed acidity, is caused by bacteria belonging to the heterofermentative group of the genus *Lactobacillus*. One hundred and ten strains of these bacteria have been isolated from wines and lees, and they have been arranged in five groups or types, according to their morphology, cultural characters, and fermentation reactions. Type I differs from previously described species, while Type III and Type IV appear to be varieties of *L. buchneri* (Henneberg emend. Pedersen), and Type II and Type V show some relationship to *L. fermentum* (Beijerinck).

The five types of bacteria bring about similar changes in the composition of fortified wines, but they differ in their tolerance of alcohol. Type I tolerates higher concentrations of alcohol than the other types, and consequently it is the type which most often causes spoilage of wines containing 20 per cent. of alcohol.

Spoilage may also be caused by the growth of some strains of bacteria in the must, before or during the primary fermentation. None of the strains studied were able to cause serious spoilage when sown in wines which contained only traces of fermentable sugar.

The bacteria are widely distributed and probably occur in practically all wineries. Since nearly all wines are exposed to bacterial contamination at some period, a wine which remains sound does so because it is not susceptible to bacterial attack.

It has been shown that the bacteria are very exacting as regards their growth requirements. The presence in the wine of accessory growth substances liberated by the autolysis of yeast cells appears to be an important factor favouring the growth of bacteria. The autolysis of yeast cells is influenced by pH and temperature, and control of these factors during the period that the wine is in contact with the yeast cells in-

fluences the susceptibility of the wine to subsequent bacterial attack.

The bacteria failed to grow in fortified wines which contained 75 to 80 parts per million of SO_2 or which had a pH below 3·6. The amounts of alcohol and tannin in the wine also have an important effect on bacterial growth. In fortified wines the five types of bacteria were killed by pasteurizing at 70° C. for one minute.

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LACTOBACILLUS Type I
Gram stained; × 1,000

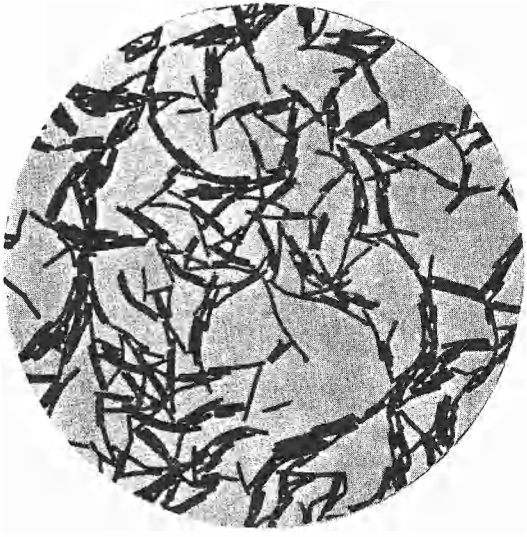

Fig. 1. 6 days at 23° C. in autolysed yeast medium (alcohol-free), after repeated subculture in this medium.

LACTOBACILLUS Type I
Gram stained; × 1,000

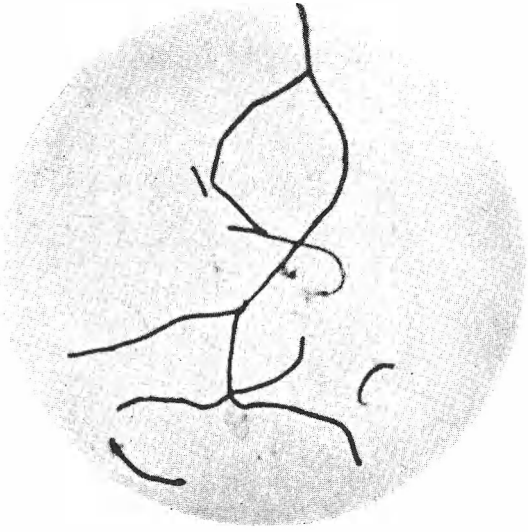

Fig. 2. 6 days at 23° C. in autolysed yeast medium containing 15 per cent. of alcohol. First subculture in this medium, after repeated subculture in alcohol-free medium.

LACTOBACILLUS Type I
Gram stained; × 1,000

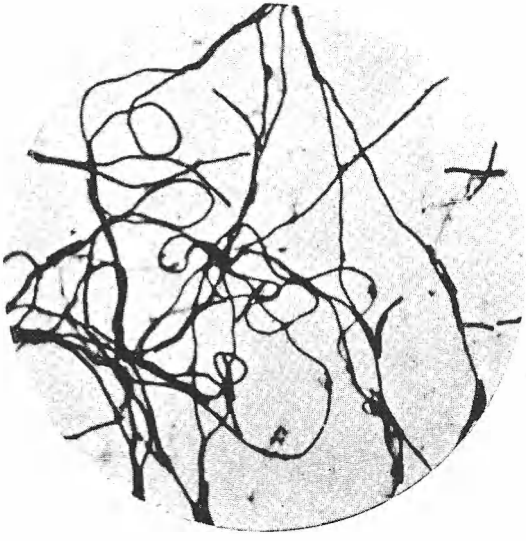

Fig. 3. 16 days at 23° C.; same type of culture as that depicted in Fig. 2.

LACTOBACILLUS Type II
Gram stained; × 1,000

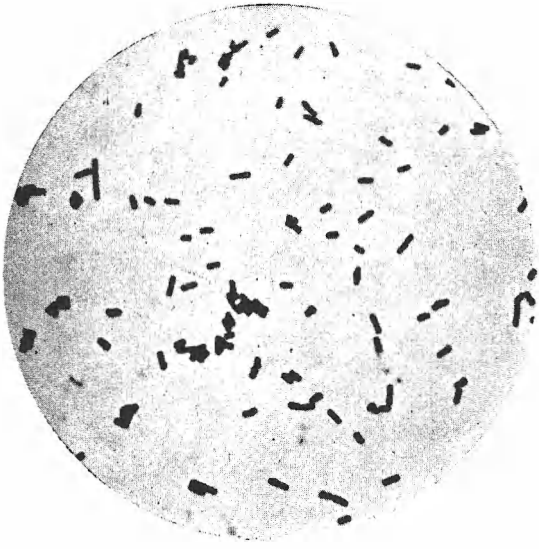

Fig. 4. 6 days at 23° C. in autolysed yeast medium.

LACTOBACILLUS Type III
Gram stained; × 1,000

Fig. 5. 6 days at 23° C. in autolysed yeast medium.

LACTOBACILLUS Type IV
Gram stained; × 1,000

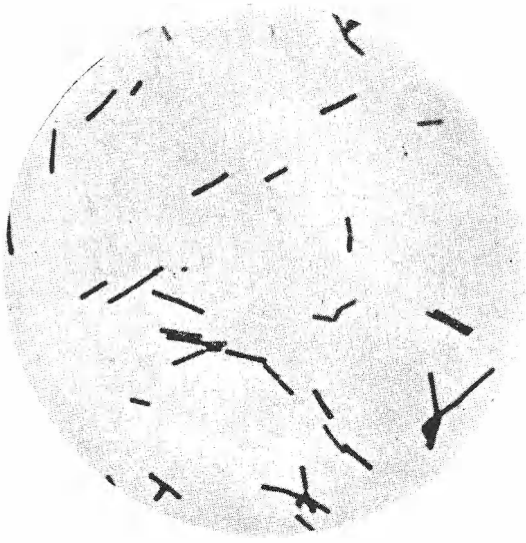

Fig. 6. 6 days at 23° C. in autolysed yeast medium.

LACTOBACILUS Type V
Gram stained; × 1,000

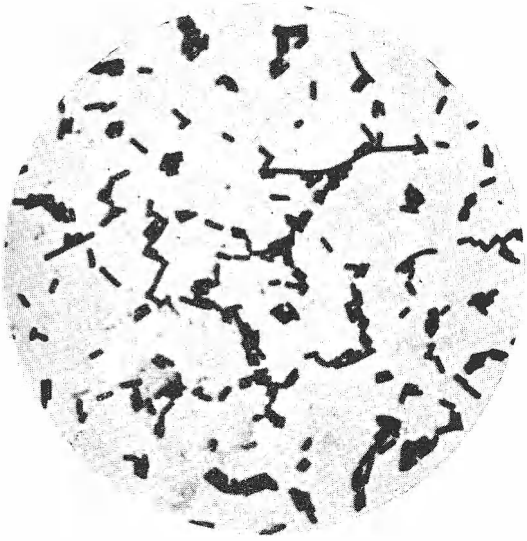

Fig. 7. 6 days at 23° C. in autolysed yeast medium.